DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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and

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VIROLOGY MANUAL

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INTRODUCTION

The purpose of a laboratory manual is to provide a standardized methodology for assay performance. Standardized methodologies minimize variability and allow for the comparison of results from laboratory to laboratory. Method standardization is a critical component of the clinical laboratory where the values obtained are used to monitor and treat patients. Unlike the research setting where the results of an experiment may be unknown, the results obtained in the clinical setting are "expected" (normal values). The clinician and clinical researcher must be assured that deviations from expected results reflect true changes due to therapy and/or disease progression of the patient and not variation in the methodology.

The first versions of this manual were circulated among the virology laboratories of the AIDS Clinical Trials Group (ACTG) as individual consensus laboratory protocols for use in ACTG clinical trials. In January 1993, these documents were compiled into a laboratory manual, which was revised and reprinted in September 1994. The distribution of the manual and its use by groups outside of the ACTG laboratories has increased significantly over the past several years. In an effort to make the manual applicable to all researchers working in the field, as well as allow for its use in the clinical laboratory, the manual was revised extensively in keeping with the guidelines recommended by the Clinical Laboratory Improvement Act.

The development of consensus assays protocol included in the manual has required a group effort involving many individuals working in the area of HIV virology. However, the compilation of this version of the manual has been accomplished by the DAIDS Virology Technologist Subcommittee. It is a manual written by the technologist for technologist. As with any effort of this magnitude, there are a few individuals who deserve special recognition: past and present chairpersons of the Virology Technologist Subcommittee - Ms. Suzy Woznick (formally University of North Carolina, Chapel Hill), Ms. Carolyn Beatty (University of Minnesota) and Ms. Joan Dragavon (University of Washington, Seattle), along with Mr. Brian Staes of the Virology Quality Assurance Contract at Rush-Presbyterian-St. Lukes Medical Center have spent many hours writing, reviewing and rewriting. Finally, many thanks to Ms. Daniella Livnat of the NIH Division of AIDS Program Office, without whose tireless commitment, encouragement and organizational skills the entire project would have never succeeded.

The manual is recognized as an official NIH publication and should be cited as follows: **Division of AIDS, National Institute of Allergy and Infectious Diseases**. 1997. DAIDS Virology Manual for HIV Laboratories. Publication NIH-97-3828. U.S. Department of Health and Human services, Washington, D.C. It is anticipated that the manual will be available electronically through the World Wide Web during 1997 and that updates and revisions will be made as necessary.

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LABORATORY PROCEDURES

BIOSAFETY

On December 6, 1991, the Occupational Safety and Health Administration (OSHA) enacted: Occupational Exposure to Bloodborne Pathogens, Final Rule: 29 CFR Part 1910-1930. The main purpose of this standard is to regulate facilities where employees could be exposed to bloodborne pathogens and to promote safe practices in an effort to minimize the incidence of disease due to these pathogens. Furthermore, it strives to reduce/eliminate occupational exposure to Hepatitis B Virus (HBV), Human Immunodeficiency Virus (HIV), and other bloodborne pathogens that employees may encounter in their workplace.

There are a number of excellent principles that should be followed when working with materials that contain bloodborne and other pathogens. These include:

It is prudent to minimize all exposure to all pathogenic organisms.

Risk of exposure to pathogenic agents should never be underestimated.

Laboratory areas should institute as many engineering and work practice controls as possible to eliminate or minimize exposure to pathogenic organisms.

As provided in 29 CFR 1910-1930, occupational exposure means reasonably anticipated skin, eye, mucous membrane or parenteral contact with blood or other potentially infectious materials that may result from the performance of an employee duty. Other potentially infectious materials may be semen, vaginal secretions, cerebrospinal fluid, amniotic fluid or any potentially infected body fluid. It may also include unfixed tissue or organs, HIV/HBV-containing cell or tissue cultures, organ cultures and their culture medium or other solution.

There are a number of areas that must be addressed in order to effectively eliminate or minimize exposure to bloodborne pathogens. The principle investigators, laboratory supervisors, and all laboratory personnel are responsible for ensuring compliance in these areas. Areas that should be included are:

The use of Universal Precautions

Establishing appropriate engineering controls

Implementing appropriate work practice controls

Using necessary personal protective equipment

Implementing appropriate housekeeping procedures

Universal Precaution Guidelines are based on the assumption that medical history and examination cannot reliably identify all patients infected with HIV, HBV, or other potentially infectious disease. Therefore, blood and body precautions must consistently be used with ALL patients and patient samples.

Engineering Controls are used to eliminate or minimize employee exposure to bloodborne pathogens. Equipment such as sharps disposal containers, hand washing sinks, biological safety hoods and special ventilated laboratory facilities are used as appropriate.

A number of Work Practice Controls help to reduce risk. Employees should wash their hands immediately after removal of gloves or other personal protective equipment. Following any contact of body areas with blood or infectious materials, employees should wash/flush their hands or exposed mucous membranes with water. Contaminated needles or other sharp objects (pipette tips, serological pipettes etc.) should be disposed of appropriately. Needles should not be recapped. Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited. Food and drink may not be kept in laboratory areas. Procedures should be conducted in such a manner to minimize splashing, spraying or generation of aerosols. Infectious materials must be placed in leak-proof containers. An appropriate biohazard warning label should be attached to any contaminated equipment. Many other aspects of work practice should be considered to minimize the possibility of exposure.

Personal Protective Equipment is one of the main lines of defense against bloodborne pathogens. Gloves, gowns, face shields/masks, safety glasses/goggles, biological safety hoods, etc. should be used consistently and properly

Housekeeping should be considered. All equipment and surfaces should be cleaned and decontaminated after contact with infectious material. Potentially contaminated broken glassware should be picked up using mechanical means (broom and dustpan). Waste containers should be maintained upright and routinely replaced and not allowed to overfill. All infectious waste must be disposed of appropriately.

Many other practices which could minimize/eliminate exposure to infectious materials should be considered according to the specifics of each workplace.

All guidelines, recommendations and descriptions above are intended as a reminder that the most important elements of creating and maintaining a safe workplace are common sense, planning, and consistency in following a plan. More complete and detailed descriptions can be found in the CDC/NIH handbook, “Biosafety in Microbiological and Biomedical Laboratories” 3rd Edition, May 1993, U.S. Department of Health and Human Services or the OSHA Handbook, “Interpretive Guidelines of the Bloodborne Pathogen Standard”, Brenda Goodner, RN, MSN, CS, Skidmore-Roth Publications.

SPECIMEN CODES

A. Primary Specimen Types

BLD - Whole Blood

BRN - Brain Tissue

CBR - Cytobrush

CRD - Cord Blood

CSF - Cerebrospinal Fluid

CVB - Cervical Biopsy/Aspirate

CVL - Cervical Vaginal Lavage

CXS - Cervical Swab

GAS - Gastric Secretions

LYM - Lymph Node Biopsy/Aspirate

OTH - Other

PLC - Placental Tissue

SAL - Saliva

SEM - Semen

SKN - Skin

SPL - Spleen

STL - Stool

TER - Tears

THR - Throat Swab

TIS - Tissue

TON - Tonsillar Biopsy/Aspirate

URN - Urine

VAG - Vaginal Swab

VSC - Vaginal Secretions

B. Derivative Specimen Types

CCP - Dry pellet from positive qualitative culture.

CDP - Culture Dry Pellet. The pelleted cells from a ministock (STK) preparation.

CEL - Cells. Patient PBMCs frozen with DMSO and stored in liquid nitrogen.

CPE - Cell Pellet from Cervix

CSU - Supernatant from Cervix

MAC - Macrophage

OTH - Other

P3P - Presumed Third Passage Isolate. Cells and medium suspension from a ministock (STK) preparation.

PCC - Previously Cultured Cells. Cells and medium collected from a qualitative culture and stored at -70^0C with no cryopreservative.

PEL - Dry Cell Pellet. Patient PBMCs from Ficoll-Hypaque separation stored as dry pellet at -70^0C.

PLA - Plasma

PLP - Presumed Low Passage Isolate. Cells and media from a quantitative micrcococulture (passage level 2).

SER - Serum

SPQ - Supernatant from quantitative micrococulture

STK - Ministock. Supernatant from quantitative micrococulture (passage level 3).

SUP - Supernatant from a qualitative culture

WBP - Whole Blood Pellet

VPE - Pellet from Vaginal Swab

VSU - Supernatant from Vaginal Swab

SPECIMEN PROCESSING

I. PRINCIPLE

The success of a protocol depends in part upon the adequate collection, preservation and retrieval of specimens. Guidelines for sample collection and storage need to anticipate the requirements of future studies which are yet to be designed and technological advances which are in the early stages of evolution. While this is not always possible, certain basic tenets exist. For example, all specimens should be collected and maintained using aseptic techniques. This includes the use of sterile tubes, pipette tips and reagents, and a work environment that is designed to prevent contamination of the sample.

II. SPECIMEN REQUIREMENTS

Consult specific protocol to determine the type and quantity of specimens required for each virologic assay.

III. REAGENTS

Dimethyl Sulfoxide (DMSO) - Store at room temperature. Use as long as it remains clear (4 - 5 years per Sigma Chemical).

Ice.

Cryoprotective Medium - Prepare enough medium for use and discard unused portion. Increase the following proportions (for 1 mL) as needed. Keep refrigerated.

0.4 mL RPMI
0.5 mL Fetal Bovine Serum
0.1 mL DMSO

For more reagents, see Qualitative PBMC Macrococulture Assay in this Manual.

Roche Specimen Wash Solution. Store at 4 - 8^{0}C and note manufacturer's outdate.

IV. EQUIPMENT AND SUPPLIES

Laminar flow hood (class 2 biosafety hood)
Gloves
Disposable lab coat
Microfuge capable of speeds > 10,000g

Centrifuge with horizontal rotor, capable of speeds up to 1800g and equipped with aerosol safe canisters
Sterile pipettes
Pipetting device
Sterile plugged pipette tips
Micropipettors at various volumes
Sterile cryopreservation vials
Sterile conical, cryopreservation vials
Sterile conical centrifuge tubes
Hemacytometer and microscope or flow cytometer for cell enumeration
-20^{0}C freezer
-70^{0}C freezer
Liquid nitrogen storage tank and boxes or canes
Surgical sponges
Betadine
60 cc syringe with 18 gauge needle
Sterile ACD solution

V. PROCEDURES

Primary Specimen Types

1. Whole Blood (BLD)

    a. Collection. Consult the specific protocol for vacutainer tube requirements.

    b. Processing.

        1) Acid Citrate Dextrose-A (ACD-A) or Heparin tubes should be processed within 4 - 6 hours of collection; consult the specific protocol for processing requirements. The tubes should be centrifuged at 1200g for 10 minutes to separate cells and plasma. The plasma is then removed avoiding the cell layer and centrifuged again at 1200g for 10 minutes to remove any contaminating cells and platelets. Plasma should then be aliquoted in sterile cryovials according to Virology Specimen Storage Recommendations in this manual or the protocol specific instructions, and stored at -70^{0}C. The PBMC are then separated as described in the Qualitative PBMC Macrococulture Assay in this manual. The PBMC can then be resuspended, counted and used for culture, stored as dry cell pellets or stored as viable cell suspensions.

        2) Citrate Cell Preparation Tubes (CCPT) should be processed within 4 - 6 hours; consult the specific protocol for processing requirements. The tubes should be centrifuged at room temperature in a horizontal rotor (swing-out

head) at a minimum of 1500g for a minimum of 20 minutes. Do not exceed 1800g, however, or some loss of cells or damage to the tube may result. After centrifugation, the plasma layer above the cells should be removed and centrifuged again at 1200g for 10 minutes to remove any contaminating PBMC and platelets. The plasma should then be aliquoted in sterile cryovials according to Virology Specimen Storage Recommendations in this manual, or the protocol specific instructions and stored at -70^0C. Add approximately 3.0 mL of PBS to the PBMC remaining in the CCPT tube, cap the tube, invert once, then remove the entire cell suspension and place into a sterile conical centrifuge tube. The cells are then washed once in calcium-free PBS and centrifuged at 400g for 10 minutes. The PBMC can then be resuspended, counted and used for culture, stored as dry cell pellets or stored as viable cell suspensions. If a CCPT tube is collected off site for next day delivery to a virology laboratory, the tube should be centrifuged within 3 hours to separate red cells and neutrophils from PBMC and plasma. The tube should then be inverted to mix the plasma and cells before placing the CCPT tube in the appropriate container for shipment. The receiving laboratory can then remove the entire layer above the gel and separate the PBMC and plasma with a 400g spin for 10 minutes. The plasma layer is removed and centrifuged at 1200g for 10 minutes to remove contaminating PBMC and platelets. The PBMC are then processed as above. This procedure of mixing the PBMC and plasma prior to shipping will result in a dilution of the plasma and should be noted. A dilution correction factor will then be used in calculating the RNA copy number. For information about training and how to purchase and receive the CCPT tubes, call Becton-Dickinson at (201) 847-4356.

2. Cord Blood (CRD)

a. Collection. After delivery of the infant, the cord is double clamped as close to the infant as possible. Cut between the clamps. After the infant is separated and while the placenta is <u>undelivered</u>, wipe the surface of the cord twice with a surgical sponge saturated in sterile saline in order to remove surface contamination with maternal blood or secretions. Prep a fairly large section of the cord, as multiple sticks may be required. The cord surface is then wiped with a betadine solution of at least 0.5% and allowed to dry for 30 seconds. Hold the cord down as low as possible to the remaining clamp. Puncture the umbilical VEIN as close to the clamp as possible, using a 60 cc syringe with an 18 gauge needle. This syringe must contain 3 mL of ACD solution prior to beginning the procedure. Aspirate venous blood until you meet resistance. Remove the syringe and raise the clamp above the puncture site. Restick the umbilical VEIN until the desired amount of blood is obtained, moving the clamp as you progress up the cord. (Some patients may be co-enrolled in other studies and may require large amounts of cord blood.)

Place 8 mL of cord blood in the appropriate CPT tubes for processing. Work must be done quickly as cord blood clots rapidly.

If the placenta is spontaneously delivered, place the placenta on a counter top and hold the cord below the surface of the counter and follow the above procedure.

Note: The collection of cord blood via the "dripping process" is not acceptable and not recommended due to increased risk of contamination between maternal and fetal blood.

b. Processing. See specific protocol for processing instructions.

3. Cerebrospinal Fluid (CSF)

a. Collection. 1-2 mL of CSF are collected in a sterile screw cap tube. Transport medium should not be used.

b. Processing. Freeze the specimen at -70^{0}C if a delay of greater than 6 hours is expected. If shipping is necessary, freeze at -70^{0}C and ship on dry ice. Vials should be labeled to indicate specific sample type.

4. Cervicovaginal Lavage (CVL)

a. Collection.

1) 10 mL of either 1x phosphate-buffered saline (PBS) or nonbacteriostatic normal saline (or normal saline) is drawn up through a plastic pipette into a 10 mL syringe. (The pipette is cut below the bulb and the remaining cath tip is inserted over the tip of a 10 mL syringe. Alternatively, a 14 gauge angiocath can be inserted over the tip of a 10 mL syringe.)

2) Introduce the pipette through the speculum into the vagina.

3) Squeeze the pipette to bathe the cervix (pipette should be directed towards the cervical os).

4) Allow the fluid to pool into the posterior fornix and aspirate into the same pipette.

5) Repeat this procedure 2-3 times with the same fluid; do not add any additional saline or PBS to the specimen.

6) Aspirate the fluid a final time and place into a cryovial for storage at -70^{0}C.

Note: Specimens should be transported to the laboratory in a timely fashion (within one hour). If this is not possible, place specimens on ice and refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

5. Endocervical Swab (CXS)

a. Collection

1) A Dacron swab is gently inserted 1 cm into the cervical os and rotated 360 degrees.

2) The swab is then placed into a sterile tube containing 2 mL of 1x PBS or non-bacteriostatic normal saline.

3) Rotate the swab 360 degrees against the inside of the tube to remove as much fluid as possible. Vortex the vial if this is possible; this procedure will aid in extracting the fluid from the swab.

4) Place the fluid in a cryovial.

5) Discard the Dacron swab.

Note: Specimens should be transported to the laboratory within one hour. If this is not possible, place specimen on ice or refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

6. Vaginal Swab (VAG)

a. Collection

1) A Dacron swab is inserted and rotated 360 degrees in all four quadrants of the vaginal vault.

2) The swab is then placed into a sterile tube containing 2 mL of 1x PBS or non-bacteriostatic normal saline.

3) Rotate the swab 360 degrees against the inside of the tube to remove as much fluid as possible. Vortex the vial if this is possible; this procedure will aid in extracting the fluid from the swab.

4) Place the fluid in a cryovial.

5) Discard the Dacron swab.

Note: Specimens should be transported to the laboratory within one hour. If this is not possible, place specimen on ice or refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

7. Other

Various tissues and body fluids may be required for protocol study. Consult specific protocols for collection and processing instructions. For example:

Brain biopsy (BRN)
Cervical biopsy (CVB)
Lymph node aspirate or biopsy (LYM)
Placental biopsy (PLC)
Saliva (SAL)
Semen (SEM)
Skin or lesion (SKN)
Spleen (SPL)
Tonsil aspirate or biopsy (TON)
Urine (URN)
Vaginal Secretions (VSC)

Derivative Specimen Types

1. Plasma (PLA)

a. CPT Tubes

Citrate Cell Preparation Tubes (CCPT) should be processed within 4 - 6 hours; consult the specific protocol for processing requirements. The tubes should be centrifuged at room

temperature in a horizontal rotor (swing-out head) at a minimum of 1500g for a minimum of 20 minutes. Do not exceed 1800g, however, or some loss of cells or damage to the tube may result. After centrifugation, the plasma layer above the cells should be removed and centrifuged again at 1200g for 10 minutes to remove any contaminating PBMC and platelets. The plasma should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C.

b. ACD or Heparin Tubes

Acid Citrate Dextrose (ACD) or Heparin tubes should be processed within 4 - 6 hours of collection; consult the specific protocol for processing requirements. The tubes should be centrifuged at 1200g for 10 minutes to separate cells and plasma. The plasma is then removed avoiding the cell layer and centrifuged again at 1200g for 10 minutes to remove any contaminating cells and platelets. Plasma should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C.

2. Serum (SER)

a. Processing. Allow the blood to clot, then centrifuge at 400-800g for 10 minutes. Serum should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C. If shipping the specimen, centrifuge as above and send at room temperature (20-24^{0}C). The serum should be aliquoted and frozen within 30 hours of collection.

3. Viable PBMC (CEL)

a. Collection. Obtain PBMC as described in Qualitative PBMC Macrococulture Assay in this manual.

b. Processing. Enumerate cells and resuspend PBMCs to a concentration of 2.5 x 10^6 to 10 x 10^6 PBMC/mL (keep on ice) with cold Cryoprotective Medium. The Cryoprotective Medium is added dropwise, with constant mixing, over 1-2 minutes. Dispense 1 mL aliquots of the cell suspension into cryovials. Place the cryovials in a small, insulated (Styrofoam), or slow-freeze container in a -70^{0}C freezer for 2-24 hours; then transfer to vapor phase liquid nitrogen (-135^{0}C) for storage.

4. Dry Cell Pellet for PCR (PEL)

a. Collection. Obtain PBMC as described in Qualitative PBMC Macrococulture Assay in this manual.

b. Processing. Aerosol resistant pipette tips should be used for this procedure. Enumerate the PBMC and adjust the sample with sterile PBS to achieve a concentration of 1-2 x 10^6 PBMCs/mL according to specific protocol instructions. The cell suspension should be dispensed into aliquots of 1 mL each in sterile cryovials according to Virology Specimen Storage Recommendations or the protocol specific instructions. Centrifuge for 3 minutes at the highest speed in a microfuge (typically >10,000 g). Aspirate the supernatant without disturbing the pellet. (Add 1 mL of Roche Specimen Wash Solution, vortex and centrifuge again for 3 minutes in a microfuge. Aspirate supernatant without disturbing pellet. If the pellet is not immediately extracted, it should be stored at -70^{0}C. Optional)

5. Whole Blood Pellet for DNA PCR (WBP)

a. Processing. Using aerosol resistant tips, add 0.5 mL of whole blood to a cryovial containing 1.0 mL of Roche Specimen Wash Solution. Cap the tube and mix by inversion several times. Incubate the sample for 5 minutes at 20-24^{0}C. Mix by inversion and incubate for 5 minutes more. Centrifuge for 3 minutes at the highest speed in a microfuge (typically > 10,000 g). Aspirate the supernatant without disturbing the pellet. Add 1.0 mL of the Roche Specimen Wash Solution to the pellet, vortex and re-centrifuge for 3 minutes as previously described. Repeat this wash step. Aspirate the supernatant without disturbing the pellet. If the pellet is not immediately extracted, it should be stored at -70^{0}C.

6. Supernatant from a Qualitative Culture (SUP)

a. Supernatant for p24 Antigen Testing - Supernatant is removed from a qualitative HIV coculture every 3-4 days as described in Qualitative PBMC Macrococulture Assay. Aliquot into a sterile cryovial and store at -20^{0}C or below until tested for p24 antigen.

b. Supernatant Collected at End of Positive Culture - When the qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, supernatant is harvested and aliquoted into sterile cryovials as described in Virology Specimen Storage Recommendations or specific protocol instructions. Store vials in vapor phase liquid nitrogen (-135^{0}C).

7. Previously Cultured Cells (PCC)

Aerosol resistant pipette tips should be used for this procedure. When a qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, the entire cell suspension is harvested and aliquoted into 1 mL samples according to Virology Specimen Storage Recommendations or specific protocol instructions. Store at -70^{0}C or below.

8. Cultured Cell Pellet (CCP)

Aerosol resistant pipette tips should be used for this procedure. When a qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, the cells and supernatant are well mixed and removed to a sterile conical tube. Centrifuge the conical tube for 10 minutes at 1400 rpm. Remove and freeze the supernatant fluid if so directed by the protocol instructions. Resuspend the cell pellet in fresh culture medium or PBS and aliquot into 4 or more sterile, conical microcentrifuge tubes. Centrifuge tubes in a microfuge at maximum speed for 3 minutes. Remove supernatant and discard. Freeze pellets at -70^{0}C or below.

9. Supernatant from a Quantitative Culture (SPQ)

Supernatant for p24 Antigen Testing - On day 14 of the quantitative HIV microculture, supernatant is removed from each well and placed in a sterile cryovial until tested for p24 antigen. Store at -20^{0}C or below.

10. Presumed Low Passage Isolate (PLP)

When a quantitative HIV microculture is positive, pool the cells and medium into a conical centrifuge tube. Mix gently, then aliquot 1.5 mL of the cell suspension into the number of vials specified by the protocol. Store at -70^{0}C or below.

11. Ministock (STK)

a. Collection. After the day 14 SPQ or PLP collection, the A1-B2 wells are fed with fresh donor cells and held to day 18. When a quantitative microculture meets the positivity criteria, supernatants and cells are harvested and stored. On day 18, remove supernatant fluids and cells from wells A1-B2 of the positive culture.

b. Processing. Pool the cells and medium into a sterile conical centrifuge tube. Centrifuge at 400-800 g for 20 minutes at 20-24^{0}C. Aliquot the supernatant medium (STK) and store at -70^{0}C. The cells remaining in the centrifuge tube can be stored as a dry pellet (see CDP processing section) or a Presumed Low Passage Isolate (see PLP processing section).

12. Cultured Cells stored as a dry pellet (CDP)

a. Collection. Cells are obtained from the ministock (STK) preparation above.

b. Processing. Wash the cells by resuspending them in approximately 4 mL of sterile PBS. Aliquot into 2-4 sterile, conical microcentrifuge vials of 1 mL each. Centrifuge in a microfuge at maximum speed for 3 minutes at 20-24^{0}C. Aspirate the PBS; store the dry pellets at -70^{0}C.

13. Other

See individual protocol for special handling of the following specimens:

Cell pellet from cervical swab (CPE)
Cell pellet from vaginal swab (VPE)
Macrophage (MAC)
Supernatant from cervical swab (CSU)
Supernatant from vaginal swab (VSU)

VIROLOGY SPECIMEN STORAGE RECOMMENDATIONS (for ACTG laboratories)

The recommendations for adult and pediatric specimen storage have been developed for Adult ACTG and Pediatric ACTG laboratories. ACTG Investigators are strongly encouraged to use storage recommendations in all new ACTG study protocols whenever possible. Their use will save freezer space, which is at a premium in most labs, and will reduce technical error.

A. Specimens from Adult Patients

Serum (SER)	1.0 mL X 5 aliquots
Plasma (PLA)	1.5 mL X 6 aliquots
Cells DMSO (CEL) Viable uncultured cells	5×10^6/0.5 mL X 4 aliquots
Qualitative Culture Supernatants Isolated from positive qualitative cultures	1.0 mL X 4 aliquots used for additional isolates as needed
Presumed Low Passage isolate (PLP) Low passage isolate from positive quantitative culture	0.5 mL X 4 aliquots
Ministocks Quantitative Cultures (STK) Isolate from positive quantitative cultures	1.0 mL X 4 aliquots
Cultured Dry Cell Pellets (CDP) Non-viable cell pellet from positive quantitative culture	4 microcentrifuge tubes (uncounted)
PCR	Whole blood processed per kit requirements in individual laboratories. Storage of PCR samples should be one of the following: (WBP) 3 Dry Pellets from 0.5 mL blood (PEL) 2 or more Ficoll Pellets 1×10^6 cells

B. Specimens from Pediatric Patients

The suggested amounts are for optimal storage and/or processing. If less blood is obtained than the 4 mL, please refer to the Priorities List on the following page for guidance.

Serum (SER)	0.5 mL X 4 aliquots
Plasma (PLA)	0.5 mL X 2 aliquots minimum, 4 aliquots

Cells DMSO (CEL) Uncultured cells - viable	5×10^6/0.5 mL X 2 aliquots (freeze in 2 aliquots if cell count is low)
Qualitative Culture Supernatant (SUP) Isolate from positive qualitative culture	1.0 mL X 4 aliquots
Dry Cell Pellets (PEL) Non-viable cells	1 whole blood pellet 0.5 mL
Presumed Low Passage Isolates (PLP) Low passage isolate from positive quantitative culture	0.5 mL X 4 aliquots
Ministocks Quantitative Cultures (STK) Isolate from positive quantitative culture	1.0 mL X 4 aliquots
Cultured Dry Cell Pellets (CDP) Non-viable cell pellet from positive quantitative culture	4 microcentrifuge tubes

PCR Whole blood processed per kit requirements in individual laboratories. Storage of PCR samples should be one of the following:

For infants less than 18 months of age:

(WBP) 3 Dry pellets from 0.3 mL blood

For infants older than 18 months of age:

(WBP) 3 Dry pellets from 1.5 mL blood
(PEL) 3 Ficoll Pellets 1×10^6 cells

C. Proposed Priorities Listing

For volumes of blood 2 mL:

Plasma: Test and store remainder
Qualitative Culture
PCR
Cells in DMSO
Dry Cell Pellets

PROTOCOL VIROLOGISTS SHOULD PROVIDE PRIORITY INSTRUCTIONS WHEN INSUFFICIENT SAMPLING IS AVAILABLE TO ACCOMPLISH PROTOCOL REQUIREMENTS.

SPECIMEN LABELING

ACTG laboratories should use specified virology laboratory management software to generate printed labels for each specimen vial, ensuring that integral protocol, patient and specimen information are included. If for some reason a vial cannot or was not labeled with a computer-generated label, a minimum of information MUST be included on the vial: unique specimen identifier, patient identifier (PID), sample collection date, and specimen type.

GUIDELINES FOR THE SHIPMENT OF SPECIMENS

GENERAL

Proper organization, packaging, shipping and handling of human bloodborne pathogens insure sample integrity while maintaining timely and safe transfer of specimens. Specific packaging and shipping procedures must be followed in accordance with federal regulations.

Do not ship on Thursdays, Fridays, weekends or the day before a holiday.

Always ship by overnight carrier. Check with your facility as to which carrier to use and for the specific labeling requirements for biohazardous shipments.

IATA regulations require that the sender notify the recipient of the dangerous goods prior to shipment. This is to alert the receiving party that the shipment is coming, and to ensure that prior arrangements have been made for someone to receive the shipment at delivery time. When contacting the recipient, include the courier company name, air bill number, and the date of expected delivery.

SPECIMENS

Specimen information, on computer disk, must accompany every shipment of patient specimens. ACTG laboratories should use appropriate virology laboratory management software to create shipping diskettes. This software should also be used to generate printed labels for each vial to ensure that integral protocol, patient and specimen information is included. If for some reason a vial cannot or was not labeled with the computer-generated label, a minimum of information MUST be included on the vial: unique specimen identifier, patient identifier (PID), sample collection date, and specimen type.

For shipments between ACTG virology laboratories, the shipping disk and vials must be arranged by PID and specimen date. The order on the shipping disk and the order of specimens in the freezer boxes must match. Shipments of greater than 15 vials must be organized in the order described above and placed in fiberboard boxes with dividers.

DANGEROUS GOODS SHIPPING REGULATIONS

Introduction:

As of January 1, 1995 the “International Air Transport Association” (IATA) implemented updated regulations governing the identification, packaging and shipping of Dangerous Goods via air transport. On January 1, 1997 the 38th Edition of the IATA Dangerous Goods Regulations becomes effective. This edition reflects the changes incorporated in the 9th Edition of the UN

Recommendations on the Transportation of Dangerous Goods, which was published in the Summer of 1995 and adopted by ICAO in the Technical Instructions for the Safe Transport of Dangerous Goods by AIR in 1997-1998. These regulations are published by the IATA Dangerous Goods Board and constitute a manual of industry carrier regulations to be followed by all IATA member airlines. Thus, freight carriers such as Federal Express, AirBorne Express and others who are members of IATA must follow these regulations as well as ensure that all customers utilizing their service adhere to them.

This document will outline the necessary regulations with which a laboratory must comply in order to safely and correctly transport items that have been identified as dangerous goods. If there is a need for more detailed instructions please refer to the latest IATA Regulation Manual.

Definition:

The IATA regulations define dangerous goods as the following:

"Dangerous goods are articles or substances which are capable of posing a significant risk to health, safety or to property when transported by air."

Classification:

Dangerous goods are further defined as those goods which meet criteria of one or more of nine United Nations (UN) hazard classes. The nine classes relate to the "type of hazard". The class of Dangerous Goods that most laboratories will be handling is **Class 6 -Toxic (poisonous) and Infectious Substances**.

Within Class 6, **Division 6.2 - Infectious Substances,** have been defined as follows:

"Infectious substances are substances known to contain, or reasonably expected to contain pathogens. Pathogens are microorganisms (including bacteria, viruses, rickettsia parasites, fungi) or recombinant microorganisms (hybrid or mutant) that are known or reasonably expected to cause infectious disease in humans or animals."

Infectious substances which are not likely to spread disease to humans or animals exposed to such are not subject to the provisions of the regulations in Division 6.2.

A second class of dangerous goods identified by the IATA regulations is **Class 9 - Miscellaneous Dangerous Goods.** This class is defined as follows:

"Substances and articles which during air transport present a danger not covered by other classes. Included in this class are: Other regulated substances, magnetized material and miscellaneous articles and substances."

The only miscellaneous article and or substance a laboratory will likely come in contact with and be responsible for shipping is **DRY ICE**. The IATA regulations state that when dry ice is utilized

as a refrigerant for dangerous goods it must be included on the **Shippers Declaration** for dangerous goods.

Packaging Material:

The IATA regulations also oversee the use and identification of appropriate packaging materials. Packaging materials must pass specified tests of quality stated in Subsection 10.5, including free drop tests, soak tests, and puncture tests. Once a manufacturer's packaging material passes the necessary testing it is assigned a package marking. This marking consists of a **U**nited **N**ations packaging symbol, the text "Class 6.2", the last two digits of the year of manufacture of the packaging material, the State (i.e., USA) authorizing the allocation of the mark, and the name of the manufacturer or other identification of the packaging specified by the appropriate national authority. An example of a UN Specification Marking is found below.

UN Symbol	Text	Year	State	Manufacturer	Complete Code
U N	Class 6.2	95	Can	SAF-T-PAK[U]	U N Class 6.2/95 Can/8-2 SAF-T-PAK

NOTE: Any packaging container that will be utilized for shipping Dangerous Goods MUST have this packaging marking displayed on the container.

The responsibility of obtaining and displaying this packaging marking on dangerous goods shipping containers is that of the manufacturer. When investigating commercial vendors for dangerous goods containers, make sure that their products have met the IATA criteria and display the appropriate UN specification markings.

Packing:

In general, the package must include the following:

1. A watertight primary receptacle (vacutainer tube, cryovial, etc.).

2. A watertight secondary packaging (plastic jar, sealed bag, sealed styrofoam container).

3. An absorbent material placed between the primary receptacle and the secondary packaging. The absorbent material, such as cotton, must be sufficient to absorb THE ENTIRE contents of ALL primary receptacles. If multiple primary receptacles are placed in a secondary packaging, they must be wrapped individually to ensure that contact between them is prevented.

4. A sturdy outside packaging container constructed of corrugated fiberboard, wood, metal or rigid plastic MUST be used. Styrofoam, plastic bags and paper envelopes are UNACCEPTABLE outer packaging. The minimum acceptable size is 7" x 4" x 2" (Fed Ex®). The tested packaging must bear the UN specification markings as required by the IATA regulation outlined above.

5. All packages containing infectious substances must contain an itemized list of contents enclosed between the secondary packaging and the outer packaging.

6. All packages containing infectious substances must be marked durably and legibly on the outside of the packaging with the NAME and TELEPHONE NUMBER OF A PERSON RESPONSIBLE FOR THE SHIPMENT.

Special Requirements:

1. *Substances shipped at ambient temperatures*: Primary receptacles may only be glass, metal or plastic. The receptacle must have a positive means of ensuring a leak-proof seal, such as heat seal, skirted stopper, or metal crimps. If screw caps are used, these must be reinforced with adhesive tape (plastic electrical tape works well).

2. *Substances shipped refrigerated or frozen (wet ice, prefrozen packs, dry ice)*: Ice or dry ice must be placed outside of the secondary packaging. The secondary packaging containing the primary receptacle (specimens) must be made secure inside by some measure of interior support (i.e.,, preformed indents in styrofoam or a cardboard support form). This will ensure that the secondary packaging remains in place after the ice or dry ice has dissipated. If wet ice is being used, the outer container must be leak-proof. If dry ice is being used then the outer container must permit the release of carbon dioxide gas. The primary receptacle must maintain its containment integrity at the temperature of the refrigerant being used as well as temperature and pressure of air transport to which the primary receptacle could be subjected if the refrigerant were to be lost.

Marking and Labeling:

The shipping laboratory (shipper) is responsible for all necessary marking and labeling of each package of dangerous goods. Each package must be of a size to accommodate all required markings and labels.

The shipper must make sure all relevant markings and labels are in the correct location and any irrelevant markings and labels are removed or obliterated. There are two types of labels required for shipment; Hazard Labels and Handling Labels. The Hazard Label identifies the class of dangerous goods and must bear the class or division number in the bottom corner of the label. The Handling Label identifies any special handling instructions pertinent to the class of dangerous goods. The following labels, required for the shipment of dangerous goods for both ambient or frozen, must be marked, durably and legibly on the outside of the package: (for examples refer to the latest IATA Regulations Guide)

1. The "PROPER SHIPPING NAME" of the content must be marked on the box with the corresponding UN number or ID number. [Example: Infectious Substance affecting humans (plasma containing Human Immunodeficiency Virus), UN 2813, 30 mL].

2. The "INFECTIOUS SUBSTANCES" hazard label.

3. The "CARGO AIRCRAFT" handling label, if applicable. If the total volume of Infectious Substance is greater than 50 mL, the package must be shipped on a cargo (non-passenger) aircraft only.

   Do NOT put the "Cargo Aircraft" handling label on packages containing less than 50 mL of Infectious Substance.

4. "MISCELLANEOUS DANGEROUS GOODS " hazard label. For frozen shipments using dry ice, the net weight of dry ice (in kilograms) must be stated on the label. There is a maximum of 200 kg per package for both passenger or cargo aircraft.

5. The NAME and TELEPHONE NUMBER of the person responsible for the shipment must be available (answered 24 hours a day) in case of an emergency regarding the package(s) shipped.

Dangerous Goods Declaration:

The shipper is responsible for the completion of the "Shippers Declaration for Dangerous Goods" form for each shipment containing dangerous goods as defined in the IATA regulations. The declaration form may be printed in black and red on white paper or it may be printed in red only on white paper. The diagonal hatchings printed in the left and right margins MUST be printed in red. The current Fed Ex® Dangerous Goods Air bill is one that is recommended as meeting these regulations and is available from Fed Ex® at no charge to the shipper.

Completion of the declaration form must be as follows:

1. The declaration must be completed in English. The English wording may be accompanied by an accurate translation in to another language.

2. There must be two copies of the declaration presented to the shipping carrier (i.e., Fed Ex®). One of these copies is held by the carrier and the other copy is forwarded with the shipment to its destination.

3. The declaration must be SIGNED by the shipper. The signature may be written by hand, or it may be in the form of a facsimile reproduced by printing or stamping. A typewritten signature is NOT ACCEPTABLE.

4. The following illustrates in detail, the necessary components required on the Shippers Declaration.

   a. Shipment Type (Delete non-applicable)

      You must strike or cross out the type of shipment that does not apply. For almost all Infectious Substance shipments, the word RADIOACTIVE is struck out, leaving the words NON-RADIOACTIVE displayed. (see below)

      NON-RADIOACTIVE

      (If radioactive substances are to be shipped refer to "Radioactive Material" Section of the latest IATA Dangerous Goods Manual.)

   b. Nature and Quantity of Dangerous Goods:

      This section of the declaration properly identifies the type and quantity of the dangerous goods that are being shipped. The example that is included below is for a shipment of dangerous goods that is classified as an Infectious Substance.

      1) Proper Shipping Name: This section must properly identify the substance that is being shipped as a dangerous goods. The use of dry ice as a refrigerant for dangerous goods must also be identified in this section of the declaration. [For example: Infectious Substance affecting humans (plasma containing Human Immunodeficiency Virus) or Dry Ice].
      2) Class or Division: This sections identifies the United Nations hazard class for each specific dangerous good. (HIV is classified as 6.2., DRY ICE is classified as 9.0)
      3) UN or ID Number: This section is an additional code that identifies the type of dangerous goods being shipped. (HIV has a UN number of UN 2814, DRY ICE has a UN number of UN 1845)
      4) Quantity and type of packing: This section identifies the type of outer packing used. As mentioned above there are certain types of outer containers that are approved by IATA (fiberboard box, wood, metal, or rigid plastic). This section must reflect the actual type of container being utilized.

         The volume or weight must be this section. (For example: 10 mL of plasma, 10 mg of tissue, or 10 Kg of dry ice). There is a volume limit affecting Infectious Substances being shipped in any one package. The IATA regulations state that a volume up to 50 mL of Infectious Substance can be packaged and shipped via passenger aircraft. If the volume of the package exceeds this, the package must be transported via cargo aircraft with a volume up to 4 liters (L). This will require the use of the "Cargo Aircraft" label on the package (see above).

5) Packing Instructions: This section identifies the specific regulations that have been provided by IATA for packaging dangerous goods. The instructions for Infectious Substances (HIV-1) are identified as 602. The instructions for dry ice, if used as a refrigerant for dangerous goods are identified as 904.

6) Additional Handling Information: The IATA regulations state that this section must be filled in with the following statement.

"Prior Arrangements as required by IATA Dangerous Goods Regulations 1.3.3.1 have been made."

Dangerous Goods Regulation 1.3.3.1 covers special arrangements required of the shipper. The shipper must notify the recipient that a dangerous goods package is being shipped. The nature of the dangerous goods must be legally shipped as specified in the IATA regulations, and has been packaged in such a manner to expeditiously transfer the package.

7) Emergency Telephone Number: The shipper must provide a telephone number that can be answered 24 hours a day in case of an emergency regarding the package(s) being shipped.

8) Name and Title of Signatory: This must identify the name and title of the individual that is responsible for signing the declaration. This information may be printed or stamped.

9) Place and Date: The date and place the declaration has been signed by the above mentioned individual. This may be printed or stamped.

10) Signature: The signature must be that of the individual for the declaration. It may be written or in a form of a facsimile reproduced by printing or stamping. A typewritten signature is NOT ACCEPTABLE!

Disclaimer:

This document is not meant as a replacement for the complete IATA regulations. It has been designed to function as a "quick-guide" to the necessary components required to ship dangerous goods under the IATA dangerous goods regulations. All specific questions or problems relating to shipping will need to be refereed to the official IATA regulations guide.

The complete IATA regulations can be found in greater detail in the latest "IATA Dangerous Goods Regulations." If you would like to obtain a copy, call 1-800-716-6326 .

CRYOPRESERVATION AND THAWING OF PBMC

I. REAGENTS

Ice

Cryoprotective Medium: RPMI 1640 containing glutamine, 10% sterile DMSO and 50% heat-inactivated fetal bovine serum (FBS). This medium should be prepared fresh for each freezing procedure and cooled to 2 to 8^0C prior to use.

Thawing Medium: RPMI 1640 containing 10% heat-inactivated FBS warmed to 20 to 24^0C.

Viability stain: 0.4% trypan blue solution, e.g.,, Sigma T8154.

Cryovials placed in ice.

II. FREEZING PROCEDURE

1. PBMC at a known concentration are centrifuged at 400 x g for 10 minutes at 20 to 24^0C and the supernatant is removed.

2. The PBMC are resuspended to a concentration of 2.5 x 10^6 to 1 x 10^7 PBMC/mL (keep on ice) with cold Cryoprotective Medium. The Cryoprotective Medium is added dropwise, with constant mixing, over 1 to 2 minutes.

3. Dispense 1 mL aliquots of the cell suspension into cryovials. Place the cryovials in a small, insulated (styrofoam) container in the bottom of a -70^0C freezer for 2 to 24 hours, then transfer to vapor-phase liquid nitrogen for storage.

III. THAWING PROCEDURE AND DETERMINATION OF VIABILITY

1. The frozen cells should be thawed rapidly in a 37^0C water bath until only a small crystal of ice remains. Cells must be handled gently to avoid mechanical injury.

2. The cells are transferred to a sterile 15-mL conical centrifuge tube following which Thawing Medium is added dropwise down the side of the tube, gently mixing with the cell suspension. Continue adding medium and mixing until the tube is filled.

3. The tube is centrifuged at 400 x g for 10 minutes at 20 to 24^0C and the supernatant is discarded. The cells are resuspended in coculture medium. An aliquot is removed and placed in PBS or Hanks balanced salt solution to yield

about 2 to 5 x 10^5 PBMC/mL. The viability is determined using 0.4% trypan blue solution. The viability should be greater than 80%.

IV. REFERENCES

Gjerset G, Nelson KA and Strong DM. Methods for cryopreserving cells, pp 61-67. *In* Manual of Clinical Laboratory Immunology, NR Rose, EC de Macario, JL Fahey, H Friedman and GM Penn (eds.), 4th ed., American Society for Microbiology, Washington D.C., 1992.

PIPETTE CALIBRATION PROCEDURES

I. PRINCIPLE

Using accurate pipettes is one of the key factors in obtaining good, reliable assay results. With time and use, pipettes become worn and less reliable. Thus, pipettes should be maintained regularly to ensure their accuracy as well as increase their longevity. Each pipette should be calibrated when it is first received and quarterly thereafter to preserve pipette accuracy. Expiration dates for the next calibration should be attached to the pipette. The precision of a pipette may be determined by making several measurements at selected settings of the pipette and calculating the coefficient of variation of those values.

One method for measuring accuracy is carried out by weighing the volume of water delivered by a pipette at selected settings and then calculating the average weight/volume. The calculated weight/volume is then compared to the theoretical weight of water at that volume.

Another method is based upon the principles defined by Beer's Law, which states that the color density of a solution is directly proportional to the concentration of a test substance or coloring reagent in that solution. A solution containing 2 grams of color dye/liter would have a color density twice as intense as a solution containing 1 gram/liter of the same dye. This principle is routinely applied in laboratories whenever colorimetric analyses are performed on laboratory samples. Blood sugar, for example, is measured colorimetrically by adding appropriate reagents to serum, and then measuring the color change intensity of the serum. The color density is directly proportional to the concentration of blood sugar in the original serum sample. A kit is available to perform a calibration of this sort. The MLA Pipette Calibration Kit contains a set of special stable dye solutions of exact concentrations. These standard solutions labeled A, B, C and O are used to prepare a standard curve for the laboratory's spectrophotometer. The Kit also contains Calibration Reagent dye of known concentration and precisely measured volumes of diluent in separate Calibration Cuvettes. A pipette is calibrated by transferring (pipetting) a quantity of Calibration Reagent dye into one of the pre-measured Calibration Cuvettes. Since the dye concentration and diluent quantity are known entities, the color intensity of the final solution in the cuvette is directly proportional to the quantity of dye pipetted.

Comparison of this color intensity to the standard curve, derived from the standard solutions contained in the Kit, will yield the volume delivered by the pipette. All measurements of color intensity for both Standard Cuvettes and Calibration Cuvettes are read in absorbance units and must be made on the same spectrophotometer.

II. SUPPLIES AND EQUIPMENT

Water	OR	MLA Pipette Calibration Kit
Weigh boats		Spectrophotometer
Analytical balance (micrograms)		Cuvette

III. PROCEDURE

A. Gravimetric Method

The calibration procedure described below for using 20 replicate measurements should be performed quarterly and on new pipettes before use. For monthly checks, use the same procedure except that only 2 readings are necessary at the appropriate volumes.

All specifications apply to measurements of water at an ambient temperature range of 18 to 25^0C. Due to evaporation of water, it is imperative that there be no delays in taking the readings, especially when calibrating volumes 100 µL. Water evaporation can lead to poor coefficient of variation values due to weight-over-time-changes. Although speed is imperative, accuracy should not be sacrificed.

1. Obtain a beaker of distilled, deionized water.

2. Place a small beaker or weighing dish on a precision microgram analytical balance. Adjust the weight to zero with the tare knob.

3. Put a pipette tip on the pipette to be calibrated. Rinse the tip 2 to 3 times with water, withdraw a sample, then dispense the water into the empty container on the balance pan.

4. Record the weight (see Appendix A), reset the pan arrest, and deliver a second aliquot of water into the container on the balance pan. Record the new weight.

5. Repeat this process 20 times recording the weight each time.

6. Calculations

    a. To determine the weight of the water in the container after each pipetting, subtract the weight of the water in the container before delivery from the pipette from the weight of the water in the container after delivery from the pipette (see Appendix A). The difference will represent the weight of the water delivered by the pipette.

    b. To calculate the mean pipetting volume, add up all the numbers in column 3 (Appendix A) and divide by 20.

    c. Calculate the standard deviation (SD) of the values in column 3.

d. Calculate precision using the coefficient of variation (relative %):

$$\frac{SD \quad X \quad 100}{Mean}$$

e. Calculate accuracy (Mean Error):

Mean Volume - Volume displayed on pipette

f. Calculate % Mean Error:

$$\frac{\text{Mean Volume - Volume Displayed}}{\text{Volume Displayed}} \quad X \quad 100$$

B. Beer's Law Method

1. Carefully open a Calibration Cuvette and place it in the work station provided at the front of this Kit. SAVE THE RUBBER STOPPER. Lay the stopper upside-down on the work station taking special care not to discard any diluent that may have adhered to the stopper.

2. Using the pipette you wish to test, withdraw one sample of diluent liquid from Calibration Cuvette. Discard this sample. If testing a pipette with disposable tips, discard the tip too.

3. Open the vial of Calibration Reagent and place it in the work station next to the Calibration Cuvette.

   Use Calibration Reagent 1 for 10-50 microliter pipettes.
   Use Calibration Reagent 2 for 51-250 microliter pipettes.
   Use Calibration Reagent 3 for 251-1000 microliter pipettes.

4. Using a fresh tip, withdraw one sample of Calibration Reagent dye, wipe the outside surface of the pipette tip, and then dispense that sample into the Calibration Cuvette. Use the pipette manufacturer's recommended pipetting technique. Replace the screw cap on the Calibration Reagent Vial. Caution: DO NOT INTERCHANGE CALIBRATION REAGENT VIAL SCREW CAPS.

5. Replace the rubber stopper on the Calibration Cuvette and thoroughly mix the solution by inverting the Cuvette 10 times. CALIBRATION CUVETTE CONTENTS MUST BE THOROUGHLY MIXED TO ENSURE KIT PRECISION.

6. Repeat steps 1-5 four times for each pipette to be tested.

7. Using the "O" Standard Cuvette, re-check the zero point of the spectrophotometer (at 515 nm) and adjust accordingly. Read the absorbance value of the pipette sample Calibration Cuvette. Wipe all cuvettes carefully with a clean dry tissue before reading the spectrophotometer.

8. Record all data on the Quality Control Sheet.

9. Use a linear regression Menu to obtain delivered volumes by inputting the absorbance values. Calculate the mean, standard deviation, and coefficient of variation.

10. The coefficient of variation (CV) must be less than 3.0%. If unacceptable, report to the supervisor for further instructions.

IV. ROUTINE MAINTENANCE

In addition to routine calibration, pipettes should be cleaned and lubricated as recommended by the manufacturer.

V. PROCEDURAL NOTES

Optimum accuracy and precision occur when pipetting aqueous liquids with moderate viscosity. The "blow out" stroke is essential for accuracy and is subject to user technique. A motorized pipette may improve accuracy and precision. Positive displacement pipettes are most useful for measurement of viscous, volatile and dense liquids. Without compromising speed and efficiency, they eliminate the possibility of aerosol contamination and subsequent cross-contamination of the samples. This is especially critical in PCR methodology.

VI. RESULTS

Guidelines for accuracy and precision measurements of pipettes:

Volume (µL)	Accuracy (Relative %)	Precision (Relative %)
0.5	≤5.0	≤3.0
1	≤2.5	≤1.5
5	≤1.5	≤0.6
10-20	≤1.0	≤0.5
50-100	≤1.0	≤0.3
200-1000	≤0.8	≤0.2
2500-5000	≤0.6	≤0.2

INITIAL AND QUARTERLY PIPETTE CALIBRATION

Pipette Brand_____Pipette Number_____ Pipette volume_____

Date_____Technician_____

(1) Sample No.	(2) Balance Reading (µg)	(3) Weight Pipetted (µg)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		

Mean Pipetting Volume = Accuracy (Mean Error) = Coefficient of Variation=

Standard Deviation = % Mean Error =

VIROLOGY ASSAY PROTOCOLS

PREPARATION OF PHA-STIMULATED UNINFECTED DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS

I. PRINCIPLE

Peripheral blood mononuclear cells (PBMC) are isolated from healthy, uninfected donor blood for use in various assays and to culture HIV. The PBMC are stimulated with the mitogen phytohemagglutinin-P (PHA-P), in the presence of human interleukin 2 (IL-2) for 24-72 hours before use to promote blast formation and replication of T-cells.

II. SPECIMEN REQUIREMENTS

Whole blood anticoagulated with heparin may be used (heparin should be at a concentration of 600 units/mL). The volume drawn is 120-240 mL.

Leukocyte concentrates (buffy coats) can be obtained from American Red Cross and is usually anticoagulated with EDTA or CPD. This is a unit of whole blood from which most of the plasma and red blood cells have been removed. The usual volume is 30 -50 mL.

For use of either of these sources of blood, the blood should be stored at room temperature and processed within 30 hours of collection.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.33 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate, or manufacturer's outdate, whichever comes first.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Observe manufacturer's outdate (one month after opening)

Sterile PHA-P - available from DIFCO desiccated in 5 mL vials (50 mg).

a. Add 5.0 mL of sterile water to vial. Mix until contents are dissolved. Add 45 mL HBSS to contents of vial. Mix well. Final concentration = 1000 µg/mL (200X concentration).

b. Divide into 1.0 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Label with date prepared and store at -20^0C. Observe manufacturer's outdate. Thaw as needed. Use 0.5 mL per 100 mL of media = 5µg/mL

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

Human IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL at -20^0C).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium = 100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see V. Procedure below.

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes
Sterile 50 mL conical tubes
Sterile 2, 5, 10, and 25 mL pipettes
Hemacytometer
25 and 75 cm^2 tissue culture flask
Sterile 500 mL bottles
Sterile 1.5 and 0.5 mL microcentrifuge tubes
20 µL, 200 µL and 1000 µL micropipettors
Sterile 200 µL and 1000 µL pipette tips
Bleach (household bleach diluted 1/100 with tap water)
Laminar flow hood (Class 2 biosafety hood)
Centrifuge capable of speeds up to 800 x g and equipped with a horizontal rotor and O- ring sealed safety cups
Compound microscope
CO_2 incubator (37 ± 1^0C with humidity)
37^0C and 56^0C water baths
Pipette aid
Sterile 60 mL syringes
Sample Site Coupler

V. PROCEDURE

1. Twice a week the lab receives blood for donor preparation. If a leukocyte concentrate prepared from a whole blood unit from the Red Cross is received, it will be tested by the Red Cross for anti-HIV, as well as hepatitis B and syphilis. Testing may not be complete when the unit is released, in which case, the Red Cross will call those results as soon as they are available. If heparinized whole blood is received it may be treated in the same way as the leukocyte concentrate, but the volume will be higher and the number of necessary tubes for white cell separation will be greater.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

2. Remove the cells from the bag, using a sample site coupler and 60 mL syringe.

3. Separate PBMC from the blood as follows:

   a. Accuspin Method: Carefully pour 20-30 mL of blood into large Accuspin tubes (as many as needed). Centrifuge the tubes at room temperature at 800 g for 20 minutes.

   b. Overlay Method: Add one part PBS or HBSS to one part blood. Blood should be carefully overlaid at a ratio of 4 parts diluted blood to 3 parts Ficoll reagent in 50 mL sterile tubes, being careful not to disturb the interface. Centrifuge tubes at room temperature at 400 x g for 30 minutes.

4. After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50 mL centrifuge tubes.

5. Wash cells by filling tubes with sterile PBS or HBSS and centrifuge at 400 x g for 10 minutes.

6. Decant supernatant after centrifugation, resuspend cells and fill tubes with sterile PBS or HBSS and wash again.

7. Resuspend each pellet in 10-30 mL of Growth Medium, depending on whether whole blood or leukocyte concentrate was used.

8. Count and record the number of viable PBMC/mL.

a. Pipette 10 µL of the sample into a 0.5 mL microcentrifuge tube, add 90 µL of trypan blue stain and mix.

b. Load a hemacytometer and count the number of PBMC in the four large cells.

c. Calculate the number of PBMC/mL: $\frac{\text{PBMC in all four squares}}{4} \times 10^5$

Example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

9. Place the cells in 75 cm^2 flasks (number of flasks depending upon workload for the week) at a concentration of 2 x 10^6/mL in Growth Medium. Total volume in each flask may be from 40-120 mL.

10. Add PHA-P at a final concentration of 5 µg/mL (e.g., 200 µL/ 40 mL medium).

11. Incubate at 37^0C, 5% CO_2 with humidity for 1-3 days before use..

VI. QUALITY CONTROL

Set up a qualitative HIV culture using the newly prepared donor PBMC as "patient cells" to verify that the new donor is HIV culture negative. (See - Qualitative PBMC Macrococulture Method.)

Do not use PHA-stimulated donor PBMC older than 3 days post stimulation.

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QUALITATIVE PBMC MACROCOCULTURE ASSAY

I. PRINCIPLE

A co-culture of peripheral blood mononuclear cells (PBMC) and uninfected PHA-stimulated PBMC are maintained under ideal conditions to allow viral reproduction in vitro. Virtually all cell cultures (>95%) from HIV-1 seropositive patients will yield detectable HIV-1 antigen by this method.

II. SPECIMEN REQUIREMENTS

The assay utilizes ACD, CPD or heparin anticoagulated peripheral blood (minimum volume of 10 to 20 mL from adults or children, 1 to 2 mL from infants). The blood must be kept at room temperature until processing and should be processed within 30 hours.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The

level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium = 100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Laminar flow hood (Class 2 biosafety hood).
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes.
Sterile 15 and 50 mL conical tubes.
Sterile 2, 5, 10, and 25 mL pipettes.
Hemacytometer
Sterile 25 cm^2 tissue culture flask.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20 µL, 200 µL, and 1000 µL pipetteman.
Sterile 200 µL and 1000 µL pipette tips.
Bleach (household bleach diluted 1/100 with tap water).
Centrifuge capable of speeds up to 1500 x g and equipped with a horizontal rotor and O- ring sealed safety cups.
Compound microscope.
CO_2 incubator (37 ± 1^0C with humidity).
37^0C and 56^0C water baths.
Pipette aid.

V. PROCEDURE

1. Log patient information into the lab computer and label specimen with the assigned specimen number.

2. Obtain PBMC as follows. Carefully label all tubes and flasks for each sample of blood being processed.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

3. Separate PBMC from the blood as follows:

    a. Accuspin Method: Carefully pour blood into Accuspin tubes. If plasma is being removed for storage, rinse original blood tubes with a volume of PBS or HBSS equal to the volume of plasma removed and add to the Accuspin

tubes. (Use 1-2 small Accuspin tubes for blood volumes less than 15 mL.) Centrifuge the tubes at room temperature at 800 x g for 20 minutes.

b. Overlay Method: If plasma is being removed for storage, rinse original blood tubes with a volume of PBS of HBSS equal to the volume of plasma removed and add back to cells and remaining plasma. If whole blood is being used, add one part PBS of HBSS to one part whole blood. Blood should be carefully overlaid at a ratio of 4 parts diluted blood to 3 parts Ficoll reagent in a 15 or 50 mL sterile tube depending on the volume of blood to be separated. Centrifuge tubes at room temperature at 400 x g for 30 minutes.

c. CPT tubes: The CPT tubes should be centrifuged at 1500 x g for 20 mins. in a horizontal rotor within 3 hrs. of collection. If the tubes are drawn off site and must be mailed, they should be centrifuged and then resuspended before shipping. They can be recentrifuged as above. NOTE: CPT tubes are made of glass and can fracture during the high speed centrifugation required for the procedure. Care should be taken when removing tubes from safety cups after spinning to avoid possible injury.

4. After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50 mL centrifuge tubes.

5. Wash cells by filling tube with sterile PBS or HBSS and centrifuge at 400 x g for 10 minutes.

6. Decant supernatant after centrifugation, resuspend cells and fill tube with sterile PBS or HBSS and wash again.

7. Resuspend the pellet in 10 mL of Growth Medium.

8. Count and record the number of viable PBMC/mL. Also record the ID number of the Donor PBMC to be used.

a. Pipette 10 µL of the sample into a 0.5 mL microcentrifuge tube, add 90 µL of Trypan blue stain and mix.

b. Load a hemacytometer and count the number of PBMC in the four large cells.

c. Calculate the number of PBMC/mL: $\frac{\text{PBMC in all four squares}}{4} \times 10^5$

Example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

Automated counting could be used, following manufacturer's procedures.

9. Set up culture as follows:

   Place a volume of patient cell suspension equal to $10x10^6$ PBMC in a labeled 25 cm^2 flask, add $10x10^6$ PHA-stimulated donor PBMC and bring the volume up to a total of 10 mL with Growth Medium. NOTE: If there are not enough patient cells, a culture may be set up using equal number of patient PBMC and donor cells at $2x10^6$ cells /mL, in a minimum volume of 5 mL per flask. Also, see Quality Control section. For very low patient cell number see Qualitative PBMC Micrococulture Procedure.

10. Incubate at 37^0C, 5% CO_2 with humidity.

11. Store remaining patient PBMC according to each protocol (viable PBMC, pellets, etc.). (See "Specimen Codes," "Specimen Processing" and "Specimen Storage Recommendations").

12. Feed cultures and harvest samples for HIV p24 antigen testing as follows:

    a. On day 3 or 4, remove 5 mL of supernatant from the flask without disturbing the cells (freeze a 1 mL aliquot for HIV p24 antigen detection) and replace with 5 mL of Growth Media. If less than 10 mL were used for the culture, remove one half the volume and replace with an equal volume of fresh medium.

    b. On day 7, remove 5 mL of supernatant and again freeze a 1 mL aliquot for testing. Replace with 5 mL Growth Media, including $10x10^6$ PHA-stimulated donor PBMC.

    c. Continue sampling and feeding in this manner until the end of culture: sampling twice per week, fresh media once per week and fresh media containing donor PBMC once per week.

13. A computer-generated sampling list is made every feeding day to identify cultures that need an aliquot of supernatant saved for testing. Save aliquots in tubes and store at -20^0C until tested for p24 antigen.

14. Maintain cultures for 21 days or until culture meets criteria for positivity.

15. At the end of culture:

    a. If the culture is negative, check an aliquot of cells for viability in order to detect a false negative due to cytotoxicity.

b. If the culture is positive, store samples according to each protocol (viable cultured PBMC, supernatant, etc.). (Please see "Specimen Processing" and "Specimen Codes.")

VI. QUALITY CONTROL

It is important to distinguish whether the priority for culturing the virus is to obtain an isolate or to make a diagnosis of HIV infection. If the latter, the standardization of patient and donor cell number and volume of media in flask is important. A positive culture is interpretable no matter how many cells were used for culture, but a negative culture may not be reliable for diagnosis if too few cells were used. A comment should be made in the computer if a nonstandard culture was set up.

VII. REPORTING RESULTS

Qualitative cultures whose supernatant p24 antigen results remain below the cutoff until at least day 21 are considered culture negative.

Qualitative cultures whose supernatant meet one of the following criteria are considered culture positive:

Two consecutive HIV p24 antigen VQA **corrected** values of > 30 pg/mL, of which the second value is at least four times greater than the first value or "out of range" (O.D.>2); or

Two consecutive HIV p24 antigen VQA **corrected** values that are "out of range" (O.D.>2); or

Three consecutive increasing HIV p24 antigen VQA **corrected** values > 30 pg/mL, where neither consecutive value is > 4 times the previous sample, but the third value is at least four times greater than the first.

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QUALITATIVE PBMC MICROCOCULTURE ASSAY

I. PRINCIPLE

Human immunodeficiency virus (HIV) has been shown to be the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). Isolation of virus specimens from AIDS patients and the presence of antibodies in their serum have helped to determine this etiologic association.

The qualitative macrococulture assay is one of the more sensitive virologic methods available for determining or verifying infectivity of peripheral blood mononuclear cells (PBMC). This procedure is usually performed in 25 cm^2 sterile flasks by coculturing 10^7 patient PBMC with an equal number of PHA-stimulated PBMC from a normal donor at a combined final concentration of 2 x 10^6 PBMC/mL. The coculture supernatant is tested twice weekly for HIV p24 antigen production which is used as a marker for viral replication. Fresh PHA-stimulated normal donor cells are added to the coculture once a week, and the culture is terminated at day 21 unless a positive result is confirmed at an earlier time interval.

In the event that an insufficient number of patients cells are available to perform a macrococulture, a qualitative micrococulture may be performed as an alternative method. The main differences in the methods are as follows:

Requires 2 x 10^6 patient PBMC's (1 x 10^6 assayed in duplicate);
Cocultured with 1 x 10^6 PHA-stimulated normal donor PBMC's;
Performed in a 24-well tissue culture plate;
5 x 10^5 fresh PHA-stimulated donor PBMC's in fresh media are added on day 7 and 14;
HIV p24 antigen is assayed on day 14 and on 21 if day 14 is negative; and
A positive culture well is defined by HIV p24 antigen levels of ≥30 pg/mL.

II. SPECIMEN REQUIREMENTS

Specimens should be stored at room temperature until processed. The assay utilizes heparinized, ACD or CPD anticoagulated peripheral blood from which PBMC are isolated by a Lymphocyte Separation Media gradient (see Methodology). Blood must be processed within 30 hours of collection. Patient PBMC s that are not used in the assay should be stored according to requirements as specified by each protocol (e.g., viable PBMC, dry pellet, etc.)

III. REAGENTS

Sterile PBS or HBSS, or Sterile 10x Dulbecco's PBS without Ca^{2+} or Mg^{2+}
Sterile water
Lymphocyte separation media (LSM or Ficoll-Hypaque)
Fetal bovine serum (FBS, sterile)

Antibiotics (e.g., Gentamicin - 10 mg/mL)
RPMI 1640 w/o L-Glutamine
L-Glutamine 200 mM
Interleukin-2 (IL-2)

Fetal Bovine Serum (FBS, sterile)

a. Thaw the 500 mL bottle of FBS completely.

b. Inactivate the FBS by immersing the bottle up to FBS level in a 56°C water bath for 30 minutes.

c. Label the bottle with the day of inactivation, and store at -20°C. Keep for up to 18 months from inactivation, or until expiration date of FBS, whichever is sooner.

Sterile 1x Dulbecco's PBS without Ca^{++} or Mg^{++}

a. Place 450 mL of distilled water into a 500 mL Nalgene bottle.

b. Add 50 mL of 10x Dulbecco's PBS without Ca^{2+} or Mg^{2+} to the bottle and mix well by inversion.

c. Open the bottle slightly and sterilize using the liquid setting on the sterilizer or sterilize by filtration through 0.22 μm Millipore filter.

d. Allow the Dulbecco's PBS to cool and label with a 6 month expiration date, or expiration of the Dulbecco's PBS, whichever is sooner and preparer's initials. Test for sterility.

e. Alternate: purchase sterile 1x PBS or HBSS

Coculture RPMI Media with 5% IL-2

a. Add 138 mL of heat inactivated FBS to a 500 mL bottle of RPMI 1640 w/o L-Glutamine.

b. Mix well by inversion.

c. Add 34.5 mL of Interleukin-2 to the mixture.

d. Add antibiotics to the mixture (e.g., 3.5 mL of Gentamicin).

e. Mix well by inversion.

f. Add 14.0 mL of 200 mM L-Glutamine to the mixture.

g. Label the bottle with the date of preparation, a 1 month expiration date, preparer's initials, and perform a sterility culture prior to use.

IV. EQUIPMENT AND SUPPLIES

Laminar flow hood (biosafety cabinet class II)
Gloves
Lab coat
Nalgene bottle, 500 mL
Sterile 50 mL conical graduated polypropylene centrifuge tubes
Sterile 15 mL conical graduated polystyrene centrifuge tubes
Sterile 75 x 100 mm tube
Serological pipettes, 10 and 2 mL
Polypropylene transfer pipettes
Disposable polystyrene blood dilution vials
Isoton II (Coulter)
Isoterge II (Coulter)
Bleach
Millipore filter, 0.22 μm
Tissue culture plate, 24-well
Pipette aid filler/controller (e.g., Drummond)
Tabletop centrifuge
Automated cell counter (e.g., Coulter Cell Counter)
Light microscope with 10x ocular
Cell dilutor (e.g., Dade dilutor)
Tissue culture incubator - 5% CO_2, 37°C and 98% humidity
56°C water bath

V. PROCEDURE

A. PATIENT SPECIMEN PREPARATION

No special patient preparation is required. Collect peripheral blood in heparin, ACD, or CPD vacutainer tube. If the patient has an abnormally low absolute lymphocyte count, the volume of blood will need to be adjusted. Do not refrigerate blood after collection. Deliver immediately to the laboratory and keep at room temperature prior to cell isolation. Isolate cells without delay (maximum, within 30 hours after collection). Log all specimens into the computer and obtain a specimen tracking number. Perform all procedures under a biological safety hood.

1. Mix the patient vacutainer tube(s) well by inverting several times.

2. Pour the whole blood into a 50 or 15 mL sterile conical centrifuge tube labeled with colored tape and the specimen log number.

3. Centrifuge at 400 x g for 10 min. at 24°C.

4. Remove the plasma which should be aliquoted into labeled tubes and stored frozen per specific protocol instructions.

5. Dilute the cells 1:2 by adding an equal volume of sterile Dulbecco's PBS.

6. Cap tubes and invert to mix several times.

7. Under or over layer the diluted blood with an equal to 2x volume of lymphocyte separation media.

8. Centrifuge at 400 x g for 30 min. at 24°C in the tabletop centrifuge.

9. Aspirate off the top layer of Dulbecco's PBS till approximately 0.5 cm above the PBMC layer.

10. Remove the PBMC layer with a sterile transfer pipette and place it into a 15 mL conical centrifuge tube.

11. Add Dulbecco's PBS in a 3:1 ratio to the cells.

12. Mix well by inversion.

13. Centrifuge the mixture for 10 min. at 400 x g at 24°C in the tabletop centrifuge.

14. Aspirate off the supernatant fraction and discard.

15. Add 10 mL of Dulbecco's PBS to the cells.

16. Resuspend the cell pellet by vigorously tapping the side of each tube with hand or by using a pipette.

17. Centrifuge the mixture for 10 min. at 400 x g at 24°C in the tabletop centrifuge.

18. Aspirate off the supernatant fraction and discard.

19. Add 1 mL coculture medium to the cell pellet.

20. Resuspend the cell pellet by vigorously tapping the side of each tube with hand or by using a pipette.

21. Remove 0.1 mL of the cell suspension into a 75 x 100 mm tube for a WBC count with an automated cell counter.

B. COCULTURE PROCEDURE

1. In two wells of a 24-well tissue culture plate, add 1 x 10^6 PHA-stimulated donor cells (see "Preparation of PHA-Stimulated Uninfected Donor Peripheral Blood Mononuclear Cells") and 1 x 10^6 patient PBMC's. Adjust final volume to 2 mL with coculture medium.

2. Put 2 mL of sterile water in corner wells to help maintain humidity.

3. Incubate at 37°C with 5% CO_2 .

4. At day 7 remove 1.0 mL of medium without disturbing cells. Replace with 1 mL fresh coculture medium containing 5 x 10^5 PHA-stimulated donor cells.

5. At day 14 remove 1.0 mL of medium without disturbing cells. Replace with 1 mL fresh coculture medium containing 5 x 10^5 PHA-stimulated donor cells.

6. Save day 14 supernatant fractions from duplicate wells separately and store at -30°C until analyzed for HIV p24 antigen by EIA.

7. Assay day 14 supernatant fractions for HIV p24 antigen and if both wells are positive, terminate culture; otherwise, continue culture until day 21.

8. Terminate culture on day 21, save supernatant fractions from duplicate wells separately, and store at -30°C until analyzed for HIV p24 antigen by EIA.

9. Assay day 21 supernatant fractions for HIV p24 antigen.

VI. RESULTS / INTERPRETATION

A. COCULTURE WELL RESULT: CRITERIA

The criteria for a positive or negative coculture well is based on VQA standardized HIV p24 antigen results obtained from the day 14 or day 21 culture samples. A culture well is considered positive if the HIV p24 antigen level is ≥30 pg/mL. A culture well is considered negative if the HIV p24 antigen level is <30pg/mL at both day 14 and day 21.

B. CULTURE RESULT: CRITERIA

The criteria for determining a positive, negative or indeterminate result for a qualitative micrococulture depends upon the HIV status of the patient.

1. For confirmed HIV-positive patients.

   If either well (or both wells) of a qualitative micrococulture is positive, the culture is considered positive. The culture is considered negative if both wells are negative.

2. For HIV-negative or HIV-undetermined patients.

   The culture is considered negative if both wells are negative and positive if both well are positive. If either well of a qualitative micrococulture is positive and the other negative, the culture is considered indeterminate and a subsequent specimen should be requested for testing.

VII. PROCEDURE NOTES

1. To reduce the chances of cross contamination and/or specimen mix-up, cells from only one patients should be set-up per 24-well tissue culture plate.

2. Laboratories performing this assay for ACTG or other DAIDS sponsored protocols, should be participating in and certified by the Virology Quality Assurance Quantitative Micrococulture certification program.

3. Cultures contain large quantities of HIV and are potentially infective to the technician handling the cultures. Gowns are required when working with any potential HIV containing specimens (i.e., peripheral blood, CSF, tissue specimens, etc.) and are changed weekly (daily if work being done in BSL-3 facility). Gloves must be worn whenever working with any potentially HIV containing specimens. Gloves should be changed often, especially if punctured or contaminated. All work must be performed in a certified biological safety laminar flow hood. All work areas in the laboratory must be wiped down with 10% sodium hypochlorite (bleach) at the beginning and end of the working day. The laminar flow hood must also be decontaminated with 10 sodium hypochlorite daily.

4. Blood collected in EDTA or CPD-A must always be diluted in saline or calcium and magnesium-free Dulbecco's PBS.

5. Lymphocyte separation media, coculture medium, and diluent used (Dulbecco's PBS or saline) must be at room temperature to prevent clumping of cells.
6. Inspect all 15 mL polystyrene and 50 mL polypropylene conical centrifuge tubes for cracks prior to use. Loss of cell suspensions will occur when cracked tubes are centrifuged.

7. Collection flask on the aspiration system must contain some bleach (i.e.,, enough to make solution 10% if full).

8. Always resuspend the cell pellet in the small quantity of liquid left after aspirating off the supernatant. Trying to resuspend cells in a larger volume of liquid, such as that added for washing, will result in a suspension of clumped cells. Recovery should be >50% (normally 90%) of retrievable cells. If the technologist is unfamiliar with lymphocyte isolation procedures, recovery should not be assessed until he or she is satisfactorily recovering >50% of the mononuclear cells. Inadequate recoveries are associated with failure to dilute the blood 1:2 with a buffer prior to layering over lymphocyte separation media, inadequate removal of cells from the plasma/lymphocyte separation media interface, failure to dilute the cells from the interface in Dulbecco's PBS, and inadequate centrifugation during the wash steps.

9. Viability of cells isolated by this method is usually 98%. Viability of cells should be assessed on all specimens by trypan blue exclusion.

10. Whole blood collected in late afternoon may be stored at room temperature and processed the next morning.

11. The level of CO_2 should be checked weekly with fyrite to determine % CO_2.

12. GLP and excellent sterile technique are very important because the cultures are maintained for up to 3 weeks and must remain free of contamination to give accurate results.

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QUANTITATIVE PBMC MICROCOCULTURE ASSAY

I. PRINCIPLE

The quantitative PBMC micrococulture assay estimates the number of infectious units of HIV per million mononuclear cells (IUPM) in peripheral blood mononuclear cells (PBMC). The greater the number of cells needed to produce a positive result, the lower the virus load in the PBMC. The assay, as described in detail below, is performed in duplicate in a 24-well tissue culture plate using six 5-fold dilutions, beginning with one million patient PBMC. Each sample of patient cells is cocultured with PHA-stimulated normal donor PBMC for 14 days. The supernatant from each individual well is assayed for viral expression of HIV-1 p24 antigen by the standard HIV p24 EIA assay.

II. SPECIMEN REQUIREMENTS

The assay utilizes PBMC isolated from heparinized, ACD, CPD or EDTA anticoagulated peripheral blood (usually 10 mL is required for adults or children and 2 mL from infants). The blood must be kept at room temperature until processing and should be processed within 30 hours of collection.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store 10X buffer at room temperature and 1X buffer at 4^0C. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium=100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes.
Sterile 15 and 50 mL conical tubes.
Sterile 2, 5, 10, and 25 mL pipettes.
Hemacytometer.
Sterile 24-well tissue culture plates.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20 µL, 200 µL, and 1000 µL pipetteman.
Sterile 200 µL and 1000 µL pipette tips.
Bleach (household bleach diluted 1/100 with tap water).
Laminar flow hood (Class 2 biosafety hood).
Centrifuge capable of speeds up to 1500 x g and equipped with a horizontal rotor and O- ring sealed safety cups.
Compound microscope.
CO_2 incubator (37 ± 1^0C with humidity).
37^0C and 56^0C water baths.
Pipette aid.

V. PROCEDURE

1. Log patient information into the lab computer and label specimen with the assigned specimen number.

2. Obtain PBMC from whole blood. See Qualitative PBMC Macrococulture Assay.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

3. Make six 5-fold serial dilutions as follows:

   a. For the first tube in the series, start with $3x10^6$ patient cells in 3 mL of Growth Medium. A minimum of $2.7x10^6$ patient PBMC (in 2.7 mL) are required for the following scheme. See note below if minimum requirement is not met.

   b. Transfer 0.6 mL of the cell suspension from step 1. to the next tube in the series containing 2.4 mL of Growth Medium. Mix.

   c. Continue as in step 2., using a new pipette tip for the removal from each tube, to make 6 dilutions. The resulting dilution scheme is 1:1, 1:5, 1:25, 1:125, 1:625, 1:3125. The resulting counts per mL will be 1,000,000, 200,000, 40,000, 8,000, 1600, and 320 patient PBMC per mL.

   NOTE: If fewer than $2.7x10^6$ but more than $2x10^6$ patient PBMC are recovered from a sample, the first tube should be adjusted to contain $2.0x10^6$ PBMC in 2.0 mL of Growth Medium. Proceed with step 2. above.

   If fewer than $2.0x10^6$ PBMC are recovered from a sample, dilute the total number of cells in 3.0 mL of Growth Medium and proceed to make the 5-fold dilutions from this starting concentration. In the computer, it will be necessary to enter the new estimated concentration per mL (total number of PBMC recovered divided by 3) rather than defaulting to $1x10^6$ for the number of cells in the 1:1 dilution.

4. Pipette 1.5 mL of sterile water into each of 4 corner wells of an appropriately labeled 24-well plate. In duplicate, pipette 1 mL of each of the 6 patient cell dilutions into respective wells. Store remaining patient PBMC according to each protocol (viable PBMC, pellets, etc.). (See “Specimen Processing” and “Specimen Storage Recommendations”.)

   NOTE: In the case of fewer than $2.7x10^6$ but $>2.0x10^6$ PBMC, the first tube will only have 1.4 mL and can only be tested in singleton. The first set of wells will include only one well with a single sample of $1x10^6$ PBMC.

5. Prepare donor cells at a concentration of $1x10^6$ cells/mL**.** One mL will be needed for each well of the plate (12) plus extra to facilitate pipetting.

   a. Centrifuge $13x10^6$ PHA-stimulated donor cells at 400 x g for 10 minutes.

   b. Decant supernatant.

   c. Mix pellet and suspend in 13 mL of Growth Medium.

6. Add 1 mL of donor cell preparation to each well.

7. Incubate at 37^0C, 5% CO_2 with humidity.

8. Feed and sample as follows:

   a. Remove 1 mL of supernatant from each well without disturbing the cells.

      1) Day 7 - discard supernatant. (These may be saved if desired, in case of trouble with the assay, but may only be used to troubleshoot the assay and not to calculate an IUPM.)

      2) Day 14 - save supernatant from each well, frozen in appropriately labeled tubes for HIV p24 antigen testing.

   b. On day 7 add 1 mL of 0.5 X 10^6 PHA-stimulated donor cells to each well.

   Prepare donor cells for feeding at a concentration of $0.5x10^6$ cells/mL**.** One mL will be needed for each well plus extra to facilitate pipetting. NOTE: This concentration differs from that used to set up the initial assay.

      1) Centrifuge $7x10^6$ donor cells at 400 x g for 10 minutes.
      2) Decant supernatant.
      3) Mix pellet and suspend in 14 mL of Growth Medium.

9. Culture wells to be sampled are listed in a "sampling list" which is computer generated each day from the laboratory management program. Supernatant aliquots are saved in sterile tubes and stored at -20^0C or -70^0C until assayed for HIV p24 antigen level.

10. IUPM is calculated by the method of maximum likelihood from the pattern of positive culture wells in the assay. A well is scored positive if the VQA corrected value 30 pg/mL.

11. At the end of culture, save the appropriate samples according to each protocol (supernatant, cells, PLP, etc.). (See "Specimen Processing" and "Specimen Codes".)

VI. REPORTING

The IUPM calculated by the computer is reported. A low Goodness of Fit (< 0.05) may invalidate the result.

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QUANTITATIVE PLASMA HIV CULTURE

I. PRINCIPLE

The Quantitative Plasma HIV Culture Assay estimates the number of infectious units of HIV per mL of plasma. Plasma is separated from whole blood by density gradient centrifugation. Serial 1:5 dilutions of plasma are cocultivated with uninfected PHA-stimulated PBMC (donor cells) in a 24-well microtiter plate for 14 days. Supernatants from each culture well are assayed for HIV p24 antigen. Infectious Units per mL (IUPM) of patient plasma are calculated based on the pattern of positive culture wells.

II. SPECIMEN REQUIREMENTS

1. Blood collected in ACD vacutainers. EDTA and heparin anticoagulants are not recommended. Plasma viremia endpoints are greater with the ACD anticoagulant and the plasma can be used for HIV RNA determination by PCR.

2. The optimum amount of blood is 10 mL; the minimum amount is 3.0 mL for pediatric specimens.

3. Cultures should be processed as soon as possible. Plasma should be removed from cells within four to six hours of collection. However, if necessary for transport, blood can be stored at room temperature for up to 30 hours. Plasma aliquots may be cultured in real-time or frozen and batch tested for quantitative viremia depending on the protocol specifications.

4. A specimen is unacceptable if it meets one of the following criteria: the blood has clotted, the specimen date is more than 30 hours old, the specimen is not labeled. If it meets any of the above criteria, notify the sender and determine the action to be taken.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store 10X buffer at room temperature and 1X buffer at 4^0C. Note manufacturer's outdate or discard one week after opening.

Penicillin - available in 5×10^6 unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^{0}C in a labeled box. Label with a 1 year outdate.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^{0}C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^{0}C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^{0}C water bath, then heat-inactivate in a 56^{0}C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^{0}C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^{0}C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^{0}C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining volume).

Acetic acid, 2% (distilled water as diluent) - store at room temperature. Discard after one year.

PHA-stimulated uninfected donor cells - see procedure for Preparation of PHA-Stimulated Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC)

Culture media:

500 mL RPMI-1640 with L-glutamine
105 mL fetal bovine serum, heat in-activated
31.0 mL Interleukin-2 (200 U/mL)
0.65 mL 1% DEAE-Dextran (optional)
Add antibiotics at recommended concentrations

Label media with reagent name, lot number, date of preparation, expiration date. Media must be stored at 4-8^{0}C, for a maximum of two weeks.

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Sterile 15 and 50 mL conical tubes and racks

Sterile 5, 10, and 25 mL pipettes
Disposable transfer pipettes
Small polypropylene ziplock bag
Hemacytometer
Sterile 24-well tissue culture plates, polystyrene
Sterile 500 mL bottles
Polystyrene round bottom tubes, 12 x 75 mm
20 µL, 200 µL, and 1000 µL micropipettors
Sterile 200 µL and 1000 µL pipette tips
Bleach (household bleach diluted 1/100 with tap water)
Laminar flow hood (Class 2 biosafety hood)
Centrifuge with safety cups and aerosol containment lids, capable of speeds up to 800 x g
Compound microscope.
CO_2 incubator (37 ± 1^0C with humidity).
37^0C and 56^0C water baths.
Pipette aid.

V. PROCEDURE

1. Invert tubes of blood several times - DO NOT SHAKE.

2. Centrifuge at 400 to 800 x g for 20 minutes at 20 to 24^0C. Transfer plasma to another centrifuge tube and recentrifuge at 800 x g for an additional 20 minutes to completely remove platelets and cell debris.

3. Prepare PHA-stimulated uninfected donor cells.

    a. Count donor cells and calculate the total number of cells in suspension.

    b. Calculate the number of mL of concentrated donor cell suspension required to provide 25 x 10^6 cells per plasma specimen to be quantitated. Add cells to culture media (total volume = 20 mL) to result in a 1.25 x 10^6 cells/mL working suspension sufficient for one quantitative plasma culture.

4. Prepare 24-well cell culture plate for quantitative plasma assay:

PLATE FORMAT

	1	**2**	**3**	**4**	**5**	**6**	
A	P	P	P	P	P	P	2 mLs of PBS in each well
B	N1	O1	P1	Q1	R1	S1	1.6 mL of donor cells/well and 400 µL/well plasma dilutions
C	N2	O2	P2	Q2	R2	S2	
D	P	P	P	P	P	P	2 mLs of PBS in each well

   a. Pipette 2.0 mL sterile PBS into each of the twelve wells in rows A and D of the cell culture plate.

   b. Seed each of the culture wells in rows B and C with 1.6 mL of the stimulated donor cell suspension.

5. Label six small polypropylene dilution tubes N through S. Dispense 960 µL culture media into each of the tubes labeled "O" through "S".

6. Add 1100 µL of the clarified plasma to tube "N".

7. Make serial 1:5 dilutions of the plasma specimen: transfer 240 µL of plasma from tube "N" to tube "O". Mix well, then transfer 240 µL of the dilution in tube "O" into tube "P". Continue this procedure: "P" to "Q", "Q" to "R", "R" to "S".

8. Dispense, in duplicate, 400 µL of the undiluted plasma (N) and each subsequent dilution (O through S) to consecutive duplicate wells. The initial dilution in the first set of wells is 1:1, i.e., undiluted ("neat") plasma.

9. Place culture plate in a small ziplock bag with culture label and seal. Label both bag and plate with specimen number, specimen type, patient ID, specimen day and technician's initials. Incubate at 37^0C in 5% CO_2 incubator with humidity.

10. On day 1, remove 1 mL of media from each well and replace with 1 mL of fresh culture media. Change pipettor tips between each well. (Plasma may sometimes be clotted in wells. Avoid removing the clotted plasma.)

11. On day 7, remove 1 mL of media from each well and replace with 1 mL of culture media containing 5 x 10^5 stimulated donor PBMCs.

12. Cultures are terminated on day 14. Remove 200 µL from each well to assay for HIV p24 antigen testing.

VI. CALCULATIONS

Each of the 12 culture wells on the quantitative plasma culture plate should be seeded with 1.6 mL of a suspension of 1.25×10^6 stimulated donor cells in culture media. To calculate the appropriate volume of concentrated donor lymphocytes:

$$\frac{\text{number of donor cells in flask}}{\text{total volume (mL) in flask}} = \frac{\text{number of donor cells required for culture}}{\text{volume of cells required for culture}}$$

VII. NOTES

The first two sets of wells often gel. This is a direct result of too much plasma in the culture. Although gel formation may occasionally occur following the addition of culture medium that contains divalent cations, this is not a universal phenomenon and the gel formation appears to be reduced in media containing DEAE-dextran. Furthermore, any gel that forms is soft and can easily be dispersed with a pipette. The remaining gel debris should not be removed. Its presence does not appear to affect virus-cell interaction or the infectivity titers.

Computer Login:

The initial volume of plasma inoculated must be recorded in the laboratory software. According to this consensus protocol, 400 microliters of undiluted plasma is aliquoted into the first set of wells (N) of the culture plate. To calculate the amount of virus present in that 400 microliter aliquot, the software program divides the IUPM result derived from the IUPM table by 0.4 milliliters. This converts the result to Infectious Units per Milliliter of plasma.

VIII. REPORTING RESULTS

A plasma culture well is scored positive if > 30 pg/mL of VQA standardized HIV p24 antigen is detected. The titer of plasma HIV is expressed as the reciprocal of the highest dilution giving a corrected HIV p24 antigen level that is > 30 pg/mL. The $TCID_{50}$ (highest dilution of patient plasma containing at least one infectious unit) is determined by the maximum likelihood applied to a simple product binomial model and is reported as infectious units per milliliter of plasma (IU/mL). The laboratory software will automatically calculate the $TCID_{50}$ in IUPM (Infectious Units per mL). In general, this correlates with the highest dilution (lowest concentration) of patient plasma giving a positive result.

IX. LIMITATIONS

Plasma viremia is not apparent in all HIV-infected patients. Infectious virus is uncommonly detected in patients with CD4 cell counts above 500. As the CD4 cell number decreases, the percentage of patients with detectable plasma viremia increases. Therefore, this assay is primarily useful for monitoring changes in plasma virus load in patients with lower CD4 cell counts and positive baseline cultures. A sustained 1-2 log reduction in the titer of the plasma is considered an indication of reduced viral load.

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HIV SYNCYTIUM-INDUCING (MT-2) ASSAY

I. PRINCIPLE

The MT-2 cell culture assay is used to detect syncytium-inducing (SI) variants of HIV. The presence of SI HIV variants has been associated with rapid progression to AIDS and a lower survival rate. The MT-2 assay is normally performed in duplicate, in 96-well flat-bottom cell culture plates. Other plate, tube or flask formats are permissible if the concentrations are adjusted accordingly.

MT-2 cells, an HTLV-I immortalized T-cell line, are cultivated with cell-free supernatants from HIV-infected PBMC cultures. Inoculated MT-2 cell cultures are monitored every 2-3 days, up to two weeks, for development of typical cytopathic effect (CPE), i.e.,, large ballooning syncytia.

II. SPECIMEN REQUIREMENTS

Supernatants from HIV-infected PBMC cultures are suitable specimens. Optimally, fresh coculture supernatants should be used, but flash-frozen supernatants are acceptable, provided they have been stored at -70^0C or lower and have not been subjected to freeze-thaw cycles. Any previously frozen supernatant should be recultured in PBMCs prior to or concurrent with the MT-2 assay to confirm viability.

III. REAGENTS

MT-2 cells: Human T-cells isolated from cord blood lymphocytes and cocultured with cells from patients with adult T-cell leukemia. (NIH AIDS Reagent Repository catalog #237).

MT-2 culture media: 500 mL RPMI 1640 supplemented with 50 mL fetal bovine serum, 10 mL penicillin (5000U/mL)/streptomycin (5000 µg/mL), and 5 mL 200 mM L-glutamine.

Positive control reference virus: NIH AIDS Reagent Repository catalog #629 (virus strain A018 or 018C).

IV. SUPPLIES AND EQUIPMENT

Sterile 96-well flat-bottom cell culture plate
Micropipettors to deliver 50, 150 and 200 µL
Sterile disposable pipette tips (Sterile ART tips may be used as an additional safeguard against contamination.)
Sterile centrifuge tubes
25 and 75 cm^2 cell culture flasks

2, 10 and 25 mL pipettes
Pipette-aid
Hemacytometer and cover slip
Inverted microscope
CO_2 incubator
Biological safety cabinet
Appropriate personal safety gear

V. PROCEDURE

A. Propagation of MT-2 cells

1. Thaw 2 vials of frozen MT-2 stock cells rapidly in 37^0C water bath.

2. Aseptically transfer cells to 15 mL centrifuge tube. Centrifuge at 600 x g for 10 minutes at room temperature. Remove DMSO-freeze media from the cells.

3. Resuspend the cell pellet in 10 mL MT-2 culture medium and transfer to a 25 cm^2 cell culture flask.

4. Incubate at 37^0C in 5% CO_2.

5. After 2 days incubation, measure cell number and viability. Split the cells when they number 10^6/mL. Seed 75 cm^2 cell culture flasks with 20 mLs at 5 x 10^5 viable cells/mL.

6. Incubate cultures at 37^0C in 5% CO_2. Split cells 1:3 every 3 to 4 days or 1:10 once each week.

B. MT-2 phenotype assay

1. Add 200 µL sterile PBS to each well designated "P" in the following diagram of a flat-bottomed 96 well cell culture plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	NEG	1	P	2	P	3	P	4	P	POS	P
C	P	NEG	1	P	2	P	3	P	4	P	POS	P
D	P	P	P	P	P	P	P	P	P	P	P	P
E	P	P	P	P	P	P	P	P	P	P	P	P
F	P	NEG	5	P	6	P	7	P	8	P	POS	P
G	P	NEG	5	P	6	P	7	P	8	P	POS	P
H	P	P	P	P	P	P	P	P	P	P	P	P

2. Harvest MT-2 cells which are growing in the log phase. For the 96-well format, prepare a 4.0 mL suspension of 3.4×10^4 MT-2 cells/mL in MT-2 media.

3. Inoculate 150 µL of the MT-2 cell suspension into each well designated with a specimen number or "NEG" or "POS". (Based on the above diagram, twenty four wells will be seeded with MT-2 cells at a final concentration of 5×10^4 cells per well.)

4. Add 50 µL of MT-2 culture media to the wells designated "NEG". Add 50 µL of positive control virus to each of the wells designated "POS". Add 50 µL of HIV-infected culture supernatant to duplicate specimen wells (1 through 8). The final volume in each well will be 200 µL.

5. Incubate the plate at 37^0C in 5% CO_2. (If the incubator is not humidified, seal the plate in a ziplock bag, then incubate.)

6. On days 3, 6, 9, 12, and 14 (plus or minus 1), use an inverted microscope to examine each MT-2 well for syncytia formation. Positive syncytia formation is defined as 3 to 5 balloons or syncytia per well. Read carefully against the negative and positive controls wells. Document the first day of observed syncytia, e.g., "SI+d6" for an isolate which was first observed to produce syncytia on day 6.

7. After the wells have been examined, use a micropipettor and disposable tips to gentle resuspend the cells in each well and then remove 130 µL of suspension. Change tips between *every* well.

8. Refeed each well with 150 µL of MT-2 culture media. Return plate to ziplock bag, if needed, and continue incubation and observation.

9. Terminate the assay on day 14. If no syncytia are observed, score the isolate as nonsyncytium-inducing (NSI).

VI. QUALITY CONTROL

1. Positive virus control must produce observable syncytia within 7 days of incubation.

2. Negative control wells must not develop syncytia. Note: minor ballooning may occur as the MT-2 cells divide, but these "balloons" are significantly smaller than in the positive wells and are not true syncytia.

3. If either control does not react as expected, the assay is suspect and should be repeated.

VII. NOTES

False negative SI results, interpreted as "NSI", can result from non-viable virus supernatant. In cases when culture supernatant viability is unknown, a control PBMC culture should be performed.

False negative SI results can also occur if the MT-2 cells are compromised. Negative SI results should be confirmed if the positive SI control requires longer than 9 days to produce typical cytopathic effect, i.e., syncytia.

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HIV DRUG SUSCEPTIBILITY ASSAY

(With Addenda I and II)

I. PRINCIPLE

The *in vitro* drug susceptibility assay measures the extent that a drug inhibits HIV p24 antigen production by PBMC acutely infected with a viral isolate. It is performed in 96-well plates with a defined previously titrated inoculum of a clinical isolate to minimize inoculum effects. The infectivity of each clinical isolate is determined prior to drug susceptibility testing using a streamlined endpoint dilution assay that is analyzed by the Spearman-Karber statistical method. Both the infectivity titration and susceptibility determination use PHA-stimulated PBMC from normal donors.

After the infectivity of a virus stock is quantified, 1000 50% tissue culture infectious doses ($TCID_{50}$) per million PHA-stimulated PBMC is used as inoculum in a second set of *in vitro* infections. Infected wells (in the absence of drug and at each of a number of drug concentrations) are refed with a 50% medium exchange after 4 days of culture, and supernatant fluid is harvested after 7 days. HIV p24 antigen is quantified and the 50% inhibitory concentration (IC_{50}) of the drug is determined using the median effect equation.

II. SPECIMEN REQUIREMENTS

Cell-free supernatant is obtained from a positive HIV culture of patient PBMC, plasma, body fluid or tissue. This may originate from a qualitative macrococulture, a qualitative microculture or a quantitative microculture. This is referred to as an “isolate” or “viral stock”. The stock from microcultures are likely to require some expansion to reach adequate levels of infectivity for the susceptibility assay. A standardized method for specimen collection/stock expansion for the drug susceptibility assay, using what are called “ministocks” has been developed (see “Specimen Processing” and “Specimen Codes”). In order to assess development of resistance to a therapeutic agent, it is optimal to test paired serial isolates in the same run. One should be from a sample collected prior to or at the beginning of therapy and the other a sample collected while on therapy or after therapy has been completed.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank’s Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer’s outdate or discard one week after opening.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer’s outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^{0}C in a labeled box. Label with a 1 year outdate, or manufacturer's outdate, whichever comes first.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^{0}C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^{0}C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^{0}C water bath, then heat-inactivate in a 56^{0}C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^{0}C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^{0}C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^{0}C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL heat-inactivated FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium=100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^{0}C for up to 1 month.

Growth Medium (culture medium) - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

Antiretroviral Drug Concentrations. Drug should be stored at -20^0C to -85^0C depending on drug (e.g., protease inhibitors should be stored at -85^0C) in borosilicate glass vials. Avoid storing drug at low concentrations.

Drug-sensitive and drug-resistant isolates can be obtained from the NIH AIDS Research and Reference Reagent Program.

IV. SUPPLIES AND EQUIPMENT

Gloves
Disposable lab coat
Laminar flow hood (Class 2 biosafety hood)
Sterile 2, 5 and 10 mL pipette
Hemacytometer
96-well, flat bottomed tissue culture plates
Sterile 1.5 and 0.5 mL microcentrifuge tubes
20 µL, 200 µL, and 1000 µL micropipettor
Sterile 50 µL, 200 µL and 1000 µL pipette tip
Multichannel 50 µL, 200 µL micropipettors
Repeat pipettor and sterile tips
Borosilicate glass tubes
1% bleach or suitable disinfectant
Low speed centrifuge with O ring sealed safety cups
Compound microscope
CO_2 incubator (37 ± 1^0C with humidity)
37^0C and 56^0C water baths

V. PROCEDURE

A. Virus Stocks: Collect cell-free, virus-containing supernatants from positive HIV cocultures (patient, drug-sensitive, and drug-resistant control viral isolates) according to standard methods found in others sections of the Manual (See "Specimen Processing" and "Specimen Codes").

NOTE: SUBSEQUENT PROCEDURE SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

B. Virus Stock Infectivity Titration: Seven serial four-fold dilutions of virus stock, ranging from 1:16 through 1:65,635, are titrated in triplicate in 96 well flat-bottomed tissue culture plates.

1. Centrifuge 1 to 3 day old PHA-stimulated donor PBMC at 400 x g for 10 minutes at 20^0C to 24^0C, remove and discard supernatant, then resuspend cells in Growth Medium and enumerate cells. Determine viability with 0.4% trypan blue exclusion dye; do not use cells if viability is less than 85%. Adjust sample with Growth Medium to a concentration of $4x10^6$ cells/mL (exactly 4.2 million cells are needed per plate as currently formatted; adjust volume accordingly). Keep in CO_2 incubator at 37^0C until step 6.

2. Add 200 µL PBS or HBSS to all wells labeled P, see "Plate Format" below.

PLATE FORMAT FOR HIV TITRATION

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
D	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
E	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

3. Add 150 µL Growth Medium to the wells labeled 4^{-3} to 4^{-8} (rows C to E, columns 4 to 9) with a multi-channel micropipettor.

4. Rapidly thaw an aliquot of the virus stock at 37^0C in a water bath until only a small crystal of ice remains. Immediately dilute the sample 1:12 in Growth Medium (e.g., 0.1 mL of virus stock to 1.1 mL of culture medium) and transfer 200 µL to each well labeled 4^{-2} (column 3 in rows C to E).

5. With a multi-channel pipette, transfer 50 µL from wells labeled 4^{-2} to wells labeled 4^{-3} (column 3 to column 4 in rows C to F). Continue such transfers, moving from *left* to *right*, changing tips prior to mixing contents of the next column of wells. Discard 50 µL from the wells labeled 4^{-8} (column 9).

6. Dispense 50 µL of PBMC (200,000 cells) from step 1 to all wells containing viral stock, moving from *right to left.*

7. Cover the plate and incubate at 37^0C, 5% CO_2 with humidity.

8. On day 4, with a multi-channel pipette, moving from *right to left* across the plate, resuspend the cells in each culture well by mixing and remove and discard 125 µL of the cell suspension. Add 150 µL of fresh Growth Medium back to each well, again moving from *right to left* . Return the plate to the incubator.

9. On day 7, the HIV titration assay is terminated and the appropriate supernatants are tested for HIV p24 antigen as follows:

    a. Transfer 100 µL of supernatant from the titration wells to wells of a HIV p24 antigen plate that contains 100 µL of Growth or Basic Medium and 20 µL of the manufacturer's disruption buffer containing Triton X-100.

    b. The assay is performed according to the recommendations of the manufacturer using the VQA reference standard and QC check samples as described in "Standard HIV p24 Antigen Assay". A well is scored "positive" if the VQA corrected value is 50 pg/mL.

10. The $TCID_{50}$ is calculated by the Spearman-Karber method. An example of this calculation follows:

a. Scoring the HIV p24 antigen plate

	1	**2**	**4^{-2}**	**4^{-3}**	**4^{-4}**	**4^{-5}**	**4^{-6}**	**4^{-7}**	**4^{-8}**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	+	+	+	+	-	-	-	P	P	P
D	P	P	+	+	-	-	-	-	-	P	P	P
E	P	P	+	+	+	-	-	-	-	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P
	r = 0	0	1	2	3	3	3	= 12				

b. Calculating $TCID_{50}$/mL

xk = dose of highest dilution
r = number of "-" responses
d = spacing between dilutions
n = wells per dilution
r = sum of r

Spearman-Karber formula: $M = xk + d [0.5 - (1/n) (r)]$
$= 8 + 1 [0.5 - (1/3)(12)]$
$= 8 + 1 (0.5 - 4.0)$

The 50% endpoint is $4^{-4.5}$
Converting to 10^x:
$x = 4.5*\log 4$
$= 4.5* 0.602$
$= 2.7$ the 50% titer is $10^{2.7}$

To calculate the $TCID_{50}$/mL of virus stock, the original dilution must be corrected by multiplying by 5 (1000 µL÷ 200 µL):

$TCID_{50}/mL = 5* 10^{2.7}$
$= 10^{0.70} * 10^{2.7}$
$= 10^{3.4}$
$TCID_{50}/mL - 2.51 \times 10^3$

C. Virus Susceptibility Testing Assay (for ZDV)

1. Prepare a 2X working solution by diluting the drug of interest in Growth Medium. (For ZDV, a 1 mM stock solution is diluted to yield concentrations of 10.0 µM, 2.0 µM, 0.2 µM, 0.02 µM and 0.002 µM in at least 1 mL of Growth Medium).

2. With a multi-channel pipette, add 200 µL PBS to all wells labeled P, (see Plate Format below).

3. With a multi-channel pipettor, add 100 µL Growth Medium to wells labeled "0" (C to E in column 3). Add 100 µL of 2X ZDV working solutions to their respective wells labeled with the 1X drug concentration (columns 4 to 8). The numbers in the illustration are the final concentrations of ZDV in µM. The remaining working solutions may be stored at -20^{0}C until day 4 or made up fresh at the time. Peptides, such as protease inhibitors, should be prepared fresh each time. (See Plate Format below.)

PLATE FORMAT FOR DRUG SUSCEPTIBILITY TESTING

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
D	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
E	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

4. Sediment 1 to 3 day old PHA-stimulated normal donor PBMC at 400 x g for 10 minutes at 20 to 24^{0}C, remove and discard supernatant, then resuspend cells in Growth Medium and enumerate cells. Adjust the sample with Growth Medium to a concentration of 4 million PBMC/mL. The viability should be determined by trypan blue exclusion and should not be used if the viability is < 85%.

5. Add 1.0 mL of these donor cells to a sterile 15 mL conical centrifuge tube and centrifuge the cells at 400 x g for 10 minutes at 20 to 24^{0}C. Remove the supernatant and add the required amount of titrated virus stock (1000 $TCID_{50}/10^6$ PBMC, therefore 4000 $TCID_{50}/4x10^6$ PBMC). The final volume should be kept to 1 mL; hence the minimum useable titer is 4000 $TCID_{50}$/mL. After mixing gently, the suspension is incubated for 1-3 hours at 37^{0}C, 5% CO_2 with humidity. Longer incubation times are recommended for lower titer virus stocks. Example Calculation:

$\frac{4000 \text{ (desired TCID}_{50}\text{/mL)}}{8000 \text{ (actual TCID}_{50}\text{/mL of virus stock)}}$ = 0.5 mL (vol. of virus stock needed)

6. After incubation, bring volume up to 2 mL for $4x10^6$ cells and dispense 100 TL of the infected cells into each well of columns 3 to 8 in rows C to E. Each well will have 0.2 X 10^6 PBMC.

7. The final volume in each well should be 200 µL. Incubate the plates at 37^0C, 5% CO_2 with humidity.

8. On day 4, examine the cells microscopically for obvious cytopathic effect (CPE), then thaw the 2X working solutions (or prepare fresh material). Add 0.5 mL of the 2X working solutions to 0.5 mL of Growth Medium to give 1X working solutions. Moving from *left to right* across the plate, mix, remove and discard 125 µL of cell suspension from the culture wells. Then add 150 µL of each of the 1X ZDV concentration (or other antiretroviral agent) working solutions to the appropriate wells (see plate format).

9. On day 7, prepare dilutions of the wells for HIV p24 antigen evaluation as follows:

   a. With a multi-channel pipettor, add 205 TL of Growth Medium, followed by 25 µL of the manufacturer's disruption buffer containing Triton X-100 to the wells in columns 1 to 6 of rows C through H of a new 96-well, flat bottomed polystyrene plate. This will be the Dilution Plate and the 36 wells just pipetted will accommodate a 1:12.5 and 1:156 dilution of the Drug Susceptibility Plate.

   b. Transfer 20 µL of supernatant from each of the drug susceptibility assay wells (rows C, D, and E of the Drug Susceptibility Plate) to their respective rows in the Dilution Plate (rows C, D, and E), using a multi-channel micropipettor (1:12.5 dilution). Mix and then transfer 20 µL from row C to row F, row D to row G, and row E to row H (total dilution of 1:156). Cover the plate with a 5 x 8 inch low-density polyethylene bag or similar product to prevent drying of the samples. Store the plate at -30^0C or lower and run the HIV p24 antigen assay within 72 hours.

10. Determine the HIV p24 antigen concentration of the diluted wells as follows:

    a. Thaw the plate with the diluted samples at ambient temperature. Transfer 20 µL from each well in rows F to H to the appropriate well or tube of the HIV p24 antigen assay kit containing 180 µL of Growth Media (final

dilution of 1:1560). A range of dilutions may need to be tested until the untreated control (0 µM drug concentration) optical densities lie on the linear slope of the calibration curve. (For example, a final dilution of 1:1000 may be better, in general, for some labs.)

b. Follow the manufacturer's procedure for HIV p24 antigen detection.

c. An extended VQA calibration curve that ranges from 400 pg/mL to 25 pg/mL in two-fold dilutions should be run. Results will be analyzed using a quadratic curve fit for the Coulter and Dupont p24 antigen assays. Analysis for the Abbott kit is not modified.

11. Calculate 50% inhibitory concentration (IC_{50}) by the median effect equation and report both raw and analyzed data as follows:

a. The median effect equation of Chou and coworkers, can be used in one of the following ways:

1) the exponential form of the equation can be used and a curve fit to the data points (F_a and drug concentration) using nonlinear regression:

Fraction affected: $(F_a) = 1/[1 + (IC_{50}/\text{drug concentration})^m]$

m = slope of the curve

Fraction affected: (F_a) = % reduction from untreated control x 0.01

Systat (Systat, Inc., Evanston, IL) can solve for IC_{50} and m simultaneously using nonlinear regression modeling. After the equation's constants (IC_{50} and m) are determined, a curve of F_a versus drug concentration can be constructed on a log-linear plot with drug concentration on the log scale of the x-axis.

2) logarithmic form of the equation can be used and a curve fit to the data points by linear regression:

$\text{Log } (F_a/F_u) = m \log [\text{drug concentration}] - m \log [IC_{50}]$

Fraction affected: (F_a) = % reduction from untreated control x 0.01
Fraction unaffected: (F_u) = % maximum x 0.01

b. "Dose effect analysis with microcomputers" by Chou and Chou (Biosoft, Ferguson, MO) calculates the IC_{50} using linear regression to solve for the best fit of the data to this logarithmic form of the median effect equation. When actual values of F_a/F_u and drug concentration are plotted on log-log

scales, the IC_{50} is the drug concentration that corresponds to y=1. The Chou and Chou software plots log (F_a/F_u) versus log (drug concentration) on linear scales, and the IC_{50} is the y intercept (since log 1 = 0). However, when using this method, F_u cannot be zero and therefore must be changed to a consistent arbitrary number if no drug effect is seen at low drug concentrations.

c. Other software can be used: (a) for nonlinear regression modeling of the exponential form of the equation, (b) for linear regression modeling of the logarithmic form of the equation, or (c) for plotting F_a versus drug concentration (or F_u versus drug concentration) after IC_{50} has been determined by solving the median effect equation.

VI. QUALITY CONTROL

A. Critical evaluation of HIV p24 antigen data derived from drug susceptibility testing is imperative.

1. A repeat infectivity titration and drug susceptibility assay should be performed if data suggest that levels of virus replication at day 7 in the virus susceptibility assay are unexpectedly either too low or too high. Each investigator will need to gain experience in recognizing these parameters that suggest extremely low or high levels of replication at day 7 in the virus susceptibility assay. Possible parameters are, as follows:

a. Suboptimal virus replication could be a possibility if an assay resulted in HIV p24 antigen levels $\leq$ 25 ng/mL in the untreated control wells. While such data may be analyzable to obtain an IC_{50}, it may not yield an IC_{50} comparable to that obtained from an assay that results in higher levels of p24 antigen.

b. The possibility that virus replication may have occurred at too high a level is suggested if evidence of early ballooning CPE is detected in drug-containing wells on day 4. In that case, the possibility should be considered that the day 7 harvest occurred after virus replication had peaked and that "breakthrough" of drug sensitive virus may have occurred in the presence of drug. For this reason, the protocol includes a microscopic examination of cultures for CPE on day 4.

B. The use of controls for donor PBMC variability in drug susceptibility testing is recommended.

1. A potential source of inconsistency in the drug susceptibility assay is the variability that is sometimes seen in the sensitivity of PBMC from different

donors to support the growth of HIV. When feasible, it is advisable to use a designated pool of donors in whose cells HIV-1 is known to replicate well. The optimum is to use the same donor's cells for infectivity titration and susceptibility testing of a particular isolate. The following optional controls can help to assess the influence of donor PBMC variability on the replicative activity of HIV-1 and the eventual calculation of IC_{50}.

a. With each batch of PBMC, inoculate 200 $TCID_{50}$ of a standard, well-characterized ZDV-resistant isolate (e.g., A018C) in triplicate wells containing 0, 0.1, and 1.0 µM ZDV. Expression of HIV-1 p24 antigen should be similar in all these wells for such a high-level ZDV-resistant isolate. Significant drug inhibition (>70% reduction at 1.0M relative to no drug, for example) raises the possibility of inadequate virus replication ("apparent" drug susceptibility) and calls into question the drug susceptibility testing using this batch of PBMC and/or the infectivity titration of the isolate.

VII. LIMITATIONS OF THE ASSAY

This assay does not discriminate reliably between ddI-susceptible and ddI-resistant isolates, or other agents not associated with at least 5 to 10 fold shifts in phenotypic drug susceptibility (e.g.,, d4T, ddC).

VIII. REFERENCES

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ADDENDUM I - HIV DRUG SUSCEPTIBILITY ASSAY WITHOUT TITRATION OF VIRAL ISOLATE

I. PRINCIPLE

ZDV drug susceptibilities of 47 viral isolates were determined in 7 laboratories using the standard method described above (HIV Drug Susceptibility Assay) and two modified methods which eliminate the titration step. In place of 1000 $TCID_{50}$ (determined by the titration step), 80-100 µL or 400-500 µL of viral stock supernatant were used to infect the donor PBMC in the Virus Susceptibility Assay. The assays were then performed as usual. When the IC_{50} from the standard and modified assays were compared, the majority of viruses were consistently classified as susceptible, intermediate or resistant by all three methods. The zidovudine IC_{50} values tended to be higher with the 400-500 µL input, therefore the lower input of 80-100 µL was chosen as a substitute for the standard 1000 $TCID_{50}$. This modification results in a faster and less expensive assay.

II. PROCEDURE

1. Omit section V.B Virus Stock Infectivity Titration from the standard procedure.

2. Begin with section V.C Virus Susceptibility Testing Assay (for ZDV). In step 5, substitute 100 µL of virus stock supernatant for the 1000 $TCID_{50}$ value that would have been added to the 4 X 10^6 donor PBMC. Continue with the standard assay as described.

III. REFERENCES

Erice A, Brambilla D, Demeter L, Penas JG, Brewster F, Stellrecht K, Reichelderfer PS, Mayers DL, Balfour HH, Japour AS, and the AIDS Clinical Trials Group Virology Committee Resistance Working Group. Simplified Susceptibility Assay for Human Immunodeficiency Virus Type 1 Clinical Isolates. Submitted.

ADDENDUM II - SIMPLIFIED HIV DRUG SUSCEPTIBILITY ASSAY

I. PRINCIPLE

Zidovudine susceptibilities for 525 clinical HIV-1 isolates were assessed using the standardized drug susceptibility assay. Zidovudine IC_{50} values were calculated both before and after reducing the number of replicates and zidovudine concentration testing in vitro. Excellent results were achieved when the 0.001 µM zidovudine concentration was omitted and when duplicate rather than triplicate wells were used at the remaining concentrations. In view of the high cost of this labor-intensive assay, it is concluded that such alterations of the existing protocol are valid and cost-effective.

II. PROCEDURE

A. In section V. C. 3. and related steps, set up the virus/drug concentration wells in duplicate instead of triplicate.

B. In section V. C. 1. and related steps, prepare and use only five concentrations of zidovudine (10.0 µM, 2.0 µM, 0.2 µM, 0.02 µM and 0 µM).

III. REFERENCES

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DRUG RESISTANCE SCREENING ASSAY
NEN Life Science Products RT-Detect™ Method

I. PRINCIPLE

Mutations in the HIV-1 reverse transcriptase (RT) gene have been shown to confer resistance to both classes (nucleoside analogs and non-nucleoside RT inhibitors) of antiviral drugs that affect RT. *In vivo*, nucleoside analogs are taken up by cells and converted to an active form before being incorporated into cDNA and causing premature chain termination. Thus for zidovudine (ZDV), the active form of the drug is ZDV-TP; for didanosine (ddI), it is ddATP. Non-nucleoside drugs must also be taken up by cells, but do not require activation to function and are thought to exert their effects directly upon the viral RT.

Because it has been reported that the RT isolated from ZDV-resistant isolates cannot be distinguished from that isolated from ZDV-sensitive isolates by studying the effect of ZDV-TP on enzymatic activity, it has become customary to determine the drug resistance of viral isolates by examining the effects of a drug on the production of viral proteins in culture. However, it appears that most RT-inhibiting drugs other than ZDV, including both activated nucleoside analogs such as ddATP and non-nucleoside inhibitors such as nevirapine do show differential effects on the activity of RTs from sensitive and resistant isolates. There have also been some recent reports that certain mutations leading to ZDV resistance cause kinetic differences in the enzyme.

The NEN Life Science Products RT-Detect™ assay (NEK-070A) is an end-point method for measuring the amount of RT activity in a sample by quantitating the amount of cDNA produced during the incubation period. When used with the NEN Life Science Products ddI resistance pack (NEK-070I), the RT-Detect™ assay can be employed with an RT-containing viral extract prepared from a single 2 mL co-culture to evaluate the amount of cDNA produced in the absence or the presence of various amounts of drug. This information can be used to calculate a 50% inhibitory concentration (IC_{50}) that numerically represents the degree of resistance of the RT to the drug. Because it includes a heteropolymeric template RNA, the RT-Detect™ kit can be used to evaluate resistance to nucleoside analogs of any base. Like other methods based on RT activity in the presence of drug, this method cannot distinguish between most ZDV-sensitive and -resistant RTs.

II. SPECIMEN REQUIREMENTS

HIV-1 is isolated by conventional co-culture techniques. This method uses 2 mL of supernatant from a 7 day co-culture.

III. REAGENTS

The NEN Life Science Products ddI Resistance Pack (NEK-070I) contains the amount of each component listed below, enough to use with two or more 96-well microplates, depending on the component. If the assay is performed as described here, the ddI Resistance Pack can be used for at least 10-12 specimens. The entire ddI Resistance Pack may be stored at -20^{0}C or below. If desired, Diluent A and 8X Buffer may also be stored at 2 -8^{0}C. Expiration dates are printed on individual component labels and on the kit box.

rHIV-1 reverse transcriptase - One (1) vial containing 50 µL of solution at 10 units/mL.

ddATP, 5 mM - One (1) tube containing 100 µL of 5 mM ddATP in 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA.

Diluent A - One (1) bottle containing 5 mL of 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA.

8X Buffer - One (1) bottle containing 2 mL of 400 mM Tris-HCl (pH 8.0) and 80 mM $MgC1_2$.

The NEN Life Science Products RT-Detect™ 2-plate kit (NEK-070A) contains the amount of each component listed below, enough to perform 192 individual reverse transcription reactions (40 µL scale) and to analyze the products on two 96-well microplates. As described, the kit can be used for 10-12 specimens if 7 levels of drug and a control are run in duplicate. The entire kit should be stored at 2 - 8^{0}C, but it is recommended to store the 20X Plate Wash Concentrate at room temperature to minimize the formation of crystals. Expiration dates are printed on individual component labels and on the kit box.

PEG Solution - One (1) bottle containing 100 mL of solution consisting of 30% (w/v) polyethylene glycol 8000 in 0.4 M NaCl.

Lysis Buffer - Two (2) bottles containing 40 mL of solution consisting of 100 mM Tris-HCl (pH 8.0), 160 mM KCl, 1 mM EDTA, 3 mM dithiothreitol (DTT), 0.3% (v/v) Triton X-100, and 10% (v/v) glycerol.

Template Solution - Four (4) tubes, each containing 1.1 mL of solution containing template RNA, primer, dNTPs, DTT, and an RNase inhibitor.

4X Buffer - Two (2) bottles containing 4 mL of solution consisting of 200 mM Tris-HCl (pH 8.0) and 40 mM $MgCl_2$.

Positive Control DNA - Two (2) tubes containing 150 µL of a synthetic oligonucleotide containing sequences complementary to both the Capture and the Detector Probes. The actual concentration of DNA (between 1000 and 3000 fmol/mL) is printed on the label.

V-Well Microplate - Two (2) 96-well microplates (one molded piece), to be used for RT reactions.

Streptavidin - Coated Microplate - Two (2) 96-well microplates (12 strips of 8 flat-bottom wells), in a re-sealable bag with a desiccant pack, to be used for the ELOSA and Detection reactions.

Hydrolysis Reagent - One (1) bottle containing 4.0 mL of alkaline solution.

Neutralization Reagent - One (1) bottle containing 4.0 mL of phosphate buffer.

Probe Diluent - Two (2) bottles containing 6.0 mL of buffer solution with salts, formamide, protein and detergent.

Probe Solution, 50X - Two (2) tubes containing 110 µL of a 50X concentrate of the HRP-labeled detector probe and the biotin-labeled capture probe.

TMB Substrate - Two (2) bottles containing 10 mL of 3, 3', 5, 5' tetramethylbenzidine (TMB) solution.

TMB Stop Solution - Two (2) bottles containing 10 mL of acidic solution.

Plate Covers - Fourteen (14) adhesive plate covers.

20X Plate Wash Concentrate - One (1) bottle containing 100 mL. Additional 20X Plate Wash Concentrate can be ordered from NEN Life Science Products (Catalog Number FP360).

Distilled or deionized water for diluting 20X Plate Wash Concentrate to 1X.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Incubator or oven capable of holding the contents at 37^0C
Automatic or manual microplate washer, vacuum source and trap, hand-held multichannel refilling syringe, or a washing bottle for washing the microplate. Blank strip wells, for use in plate washers with less than a full plate of test wells, are available from NEN Life Science Products on special request
Disposable absorbent bench top paper and paper towels
Vortex mixer
Micropipettor(s) capable of accurately delivering 1 - 1000 µL
Reagent reservoirs for reagent preparation and additions, such as Costar Catalog Number 4870. Due to small reagent volumes, not for use with Reaction Mix or ELOSA Mix
Microplate reader with the capability to read absorbance at 450 nm. Use of a 650 nm reference filter is recommended

Liquid household bleach for inactivation of biohazardous specimens
Polypropylene tubes for preparing dilutions of standards
Centrifuge and tubes for sample processing

V. PROCEDURE

A. Preparation of viral extract

1. Centrifuge 2 mL of supernatant from a 7 day co-culture at 1500 x g for 10 minutes to pellet cells and debris.

2. Add 1 mL of PEG Solution to the supernatant and keep at 4^0C overnight.

3. Centrifuge at 2100 x g for 45 minutes to prepare a viral pellet.

4. Resuspend the pellet in 300 µL of Lysis Buffer. This solution contains the RT from the isolate. If desired, the solution may be stored at -70^0C for at least 1 month before completing the assay.

B. Specific steps for measuring resistance to ddI

Testing for ddI resistance requires running a single RT sample in the presence and absence of ddATP (Ahluwalia, et al., 1987). With the exception of the modifications described below, the assay is identical to that described in the RT-Detect™ kit manual and you must be familiar with that procedure in order to perform a test for ddI resistance. Before running the ddI resistance assay for the first time, review the "Limitations of Procedure" (Section VII).

Differences from the RT-Detect™ kit manual

1. For ddI resistance, the 4X Buffer supplied in the RT-Detect™ kit is not used. Instead, 5 µL of the 8X Buffer from the ddI Resistance pack and 5 µL of a ddATP dilution are added. Thus a reverse transcriptase reaction for ddI resistance contains the following components: 25 µL of Reaction Mix (consisting of 20 µL of Template Solution and 5 µL of 8X Buffer), 5 µL of solution containing a known concentration of ddATP, and 10 µL of a standard RT or a sample containing RT.

2. Because many viral isolates contain too little RT to reliably measure in a 1 hour reaction, the reverse transcription reactions should be incubated for 24 hours.

3. Each RT sample must be run at least in the presence and absence of ddATP. Running a number of ddATP concentrations as described below provides enough data to permit the calculation of an IC_{50} via the median effect equation (Chou and Talalay, 1984; Chou, 1991).

4. Preparation of Solutions for Day 1 - ddI resistance

a. ddATP dilutions

The procedure described below uses 2 strips of wells per sample to generate a 7-point inhibition curve in duplicate. This amount of information permits the calculation of an IC_{50}. To run fewer points, or compare to a cutoff value, adjust the number and the preparation of ddATP dilutions accordingly to prepare the necessary volume.

To prepare enough of each ddATP dilution for one full plate of wells, make a series of ddATP dilutions in Diluent A and label them as shown in the following table.

Label	Drug Added	Diluent A	µM in Tube	µM in Assay
H	40 µL of ddATP, 5M	210 µL	800	100
G	50 µL of H	150 µL	200	25
F	50 µL of G	150 µL	50	6.25
E	100 µL of F	100 µL	25	3.12
D	100 µL of E	100 µL	12.5	1.56
C	50 µL of D	150 µL	3.12	0.39
B	50 µL of C	150 µL	0.78	0.09
A	None	100 µL	0	0

b. Reaction Mixes - ddI

Set up 8 microcentrifuge tubes labeled A', B', C', D', E', F', G' and H' to correspond to the tubes A through H that hold the ddATP dilutions. For each RT sample to be run, add:

48 µL of Template Solution
12 µL of 8X Buffer
12 µL of the correspondingly-labeled ddATP solution to a labeled microcentrifuge tube. Thus, for an experiment with 3 samples and the rHIV RT control, Reaction Mix A, for example, will contain 192 µL of Template Solution, 48 µL of 8X Buffer and 48 µL of ddATP Solution A.

C. Specific steps for measuring resistance to Nevirapine

Although the ddATP and Diluent A are not used, it is still convenient to use the 8X Buffer and rHIV-1 RT from the ddI Resistance Pack as a supplement to the RT-Detect™ kit to measure resistance to nevirapine. At the time of this writing, nevirapine has just become commercially available and it is not included in the kit. With the exception of the modifications described below,

the assay is identical to that described for ddI resistance and you must be familiar with that procedure in order to perform a test for nevirapine resistance. Before running the nevirapine resistance assay for the first time, review the "Limitations of Procedure" (Section VII).

Differences from measuring ddI resistance

1. Due to solubility limitations, Nevirapine must be dissolved and diluted in DMSO, not in Diluent A.

2. In order to minimize the amount of DMSO introduced into the RT reactions (DMSO can be inhibitory in large enough amounts) only 2 µL of Nevirapine dilution is added to each Reaction Mix. This alters the amounts of solutions added to prepare the Reaction Mixes.

3. Each RT sample must be run at least in the presence and absence of nevirapine. Running a number of Nevirapine concentrations as described below provides enough data to permit the calculation of an IC_{50} via the median effect equation (Chou and Talalay, 1984; Chou, 1991).

4. Preparation of Solutions for Day 1 - Nevirapine resistance

   a. Nevirapine dilutions

   The procedure described below uses 2 strips of wells per sample to generate a 6-point inhibition curve in duplicate wells. This amount of information permits the calculation of an IC_{50}. To run fewer points, or compare to a cutoff value, adjust the number and the preparation of Nevirapine dilutions accordingly to prepare the necessary volume.

To prepare enough of each Nevirapine dilution for one full plate of wells, prepare a 100 mM stock solution of Nevirapine in DMSO and prepare further dilutions in DMSO as shown in the following table.

Label	Drug Added	DMSO	µM in Tube	µM in Assay
G	18 µL of 100 mM Nevirapine	82 µL	18000	375
F	10 µL of G	90 µL	1800	37.5
E	10 µL of F	90 µL	180	3.75
D	10 µL of E	90 µL	18	0.375
C	10 µL of D	90 µL	1.8	0.038
B	10 µL of C	90 µL	0.18	0.004
A	None	90 µL	0	0

   b. Reaction Mixes - Nevirapine

Set up 8 microcentrifuge tubes labeled A', B', C', D', E', F', G' and H' to correspond to the tubes A through H that hold the nevirapine dilutions. For each RT sample to be run, add:

48 µL of Template Solution
12 µL of 8X Buffer
10 µL of water
2 µL of the correspondingly-labeled Nevirapine solution to a labeled microcentrifuge tube. Thus, for an experiment with 3 samples and the rHIV RT control, Reaction Mix A, for example, will contain 192 µL of Template Solution, 48 µL of 8X Buffer, 40 µL of water and 8 µL of Nevirapine Solution A.

D. Assay Day 1

1. Preparation of Solutions

a. rHIV-1 RT

Prepare a standard rHIV-1 RT dilution as follows: Add 5 µL of starting RT (supplied at 10 units/mL) to 95 µL of Lysis Buffer. Mix, and then add 10 µL of the dilution to 240 µL of Lysis Buffer to give a solution containing 0.02 units/mL of RT. Use this final solution (0.02 units/mL) as the standard drug-sensitive RT dilution for the test.

b. Samples

Culture supernatant samples (2 mL) are processed with PEG to give a viral pellet and resuspended in Lysis Buffer (300 µL) as described above. The Lysis Buffer samples are used directly without dilution for resistance assays.

c. Drug Solutions and Reaction Mixes. Described above.

2. Procedure

a. For each sample or RT standard to be run, add 30 µL of the appropriate Reaction Mix A'- H' to 2 wells in the correspondingly-labeled row of the "v"-well plate. That is, Reaction Mix A' goes into plate row "A", etc. Be careful not to confuse Reaction Mix A' with drug dilution A, etc. Note that there are only 7 Reaction Mixes described for Nevirapine, so one row will either be empty, or can be used to run additional standards or samples.

b. Add 10 µL of each sample or RT standard to 16 wells in two columns of the plate. Each RT sample should be in 2 columns of wells and each plate

row should have a different concentration of drug. Cover the plate, sealing carefully to minimize evaporation. Incubate for 24 hours at 37^0C.

E. Assay Day 2

1. Preparation of Solutions

a. ELOSA Mix

For each sample to be run, add 18 µL of Probe Solution and 882 µL Probe Diluent to a conical centrifuge tube to prepare ELOSA Mix.

b. Plate Wash

Prepare Plate Wash by warming 20X Plate Wash Concentrate at 37^0C if necessary to dissolve any crystals and then diluting it to 1X with distilled or deionized water. About 500 mL of 1X Plate Wash is needed to prime an automated washer and run one microplate. 1X Plate Wash should be made fresh daily.

2. Procedure

a. Denaturation (and Hydrolysis of Template RNA)

1) Remove the plate cover and add 10 µL of Hydrolysis Reagent to each well. Cover the plate and incubate 15 minutes at 37^0C.

2) Add 10 µL of Neutralization Reagent to each well.

b. Sandwich Hybridization

1) Transfer all the liquid from each v-well to a corresponding well in the flat-bottom streptavidin-coated plate. If the plates were tightly sealed, there should be about 40-50 µL of liquid in each well. At this step, volume variations do not affect the assay results.

2) Add 50 µL of ELOSA Mix to each well. Cover, and incubate for 2 hours at 37^0C.

c. Detection

1) Wash the plate 6 times with 1X Plate Wash.

2) Add 100 µL of TMB Substrate to each well. Cover, and incubate 1 hour at room temperature.

3) Add 100 µL of Stop Solution to each well. Within 1 hour, read the absorbance twice, once at 450 nm and once at 490 nm, using a 650 nm reference filter in each case if possible.

VI.. CALCULATION OF IC_{50}

A. Signal Requirements

1. If the absorbance of a sample in row A (no drug) is less than 0.500, there is insufficient RT in the sample to give a reliable IC_{50}. Before running the resistance assay on a large set of samples, it may be wise to check the total RT activity of each sample in a preliminary experiment to be sure it is sufficient to perform the test. In a test with 25 clinical isolates, 3 (12%) had insufficient activity to perform the test.

2. If the absorbance of a sample in row A is off-scale at 450 nm (for example, greater than 4.000 on a Molecular Devices plate reader), the absorbance reading at 490 nm (if it is on-scale) may be used to calculate the results. If the absorbance reading at 490 nm is off-scale, the remaining RT sample should be diluted 20-fold with Lysis Buffer and used to repeat the test.

3. If the absorbance of a sample in row A is between 0.500 and 4.000 (or other limit of the plate reader used) at 450 nm, use the readings at 450 nm to calculate the results.

B. Calculations

1. Average the absorbance values for replicate tests to get the mean absorbance for each.

2. Divide the mean absorbance values for each RT sample by the mean absorbance of the same reaction in row A (no drug) to normalize the results for the amount of RT activity in the sample.

3. Plot the normalized mean absorbance for each RT reaction on the y-axis versus the log of the concentration of drug (µM) in that reaction on the x-axis. Examining the plot can help to identify and delete outlying points. Sometimes one of the two duplicate wells is an outlier, but the other well can be used.

4. If a sufficient number of drug concentrations were run to obtain a reasonable curve, commercially available software such as Systat (Systat, Inc.) or JMP (SAS Institute, Inc.) may be used to calculate an IC_{50} (the concentration of drug that inhibits the activity of the RT sample by 50% relative to the activity in the absence of drug) by the median effect equation (Chou and Talalay, 1984; Chou, 1991):

$$FA = 1 - \frac{1}{1+(Y/Dose)^m}$$

where: FA = (0.01)(% reduction from untreated control)
DOSE = drug (µM) in the RT reaction
$Y = IC_{50}$
M = slope

VII. QUALITY CONTROL

The user must establish any cut-off value to be used in distinguishing drug-sensitive from drug-resistant isolates.

VII. Limitations of Procedure

A. Safety Considerations

In the US only, the components of 8X Buffer are considered to be irritating to eyes and skin.

HYDROCHLORIC ACID; 2-CHLOROACETAMIDE;
FORMAMIDE; POTASSIUM HYDROXIDE
DANGER
CONTAINS MATERIALS THAT CAN BE FATAL IF INHALED, SWALLOWED, OR ABSORBED THROUGH SKIN. CONTAINS CORROSIVE MATERIALS THAT CAN CAUSE SEVERE BURNS TO EYES, SKIN AND ALL OTHER BODY TISSUES. CONTAINS MATERIALS THAT ARE POSSIBLE MUTAGENS AND TERATOGENS. TARGET ORGANS: Skin, Eyes, Male Reproductive Systems, Thyroid, Ovaries, Fallopian Tubes, Lungs, Gastrointestinal Tract, Blood, and Mucous Membranes. Do not get in eyes, skin, or clothing. Avoid breathing vapors. Use adequate ventilation. Wash thoroughly after handling. FIRST AID: In case of contact, immediately flush eyes and skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes.

1. Handle assay specimens, especially those of human origin, as if capable of transmitting an infectious agent (NIH Guidelines, BSL 2 or higher, as necessary, [U.S. Department of health and Human Services, 1988]).

2. Wipe spills promptly with 1% sodium hypochlorite solution (1:5 dilution of liquid household bleach). Contaminated materials should be disposed of as biohazardous waste.

3. Do not pipette by mouth. Avoid splashing and generation of aerosols.

4. Do not eat, smoke, or drink in areas in which specimens or kit reagents are handled.

5. Wear disposable gloves throughout the test procedure. Dispose of the gloves as biohazardous waste. Thoroughly wash hands afterward.

B. Performance Considerations

1. This method is not useful for determining resistance to ZDV.

2. **Prevent RNase Contamination** – The template RNA provided in the RT–Detect™ kit can be degraded by RNase. The Template Solution contains an RNase inhibitor, so RNase contamination will normally not be a problem in the assay. Two simple precautions can minimize RNase contamination if employed during the steps prior to the addition of the Hydrolysis Reagent. (Since the Hydrolysis Reagent destroys RNA, it is not necessary to take precautions to avoid RNase contamination in subsequent steps.) Wearing gloves at all times when working with RNA will prevent RNase contamination originating from the skin. Use of sterile disposable polypropylene plasticware, especially pipette tips, is recommended to prevent RNase contamination originating from labware.

3. There is not a sufficient amount of the components of the Reaction Mix and ELOSA Mix in the kit to allow preparing enough excess to permit the use of a trough and a multichannel pipette for addition of these reagents. The use of a repeater pipettor is recommended for these steps.

4. Before or after adding it to the microplate, avoid placing the TMB Substrate in proximity to a container of diluted bleach, such as is commonly used for decontamination in biosafety hoods. Vapors from the bleach solution can cause color development by the TMB, even if the plate is covered and kept in a hood.

5. Do not allow the microplate wells to dry out once the assay has begun.

6. Do not use kit components beyond the expiration date. This date is printed on individual component labels and on the kit box.

7. Do not substitute reagents from other kits. Reagents have been optimized for performance with each kit lot. Dilution or alteration of reagents may result in loss of sensitivity or other undesirable changes in kit performance.

8. Do not interchange vial or bottle caps and stoppers: this will lead to cross-contamination of reagents. Use specific reservoirs for specific reagents, and use

clean pipettes or pipette tips for each reagent. Cross-contamination of samples could cause false results.

9. Incubation times or temperatures other than those specified may give erroneous results.

C. General Precautions

1. Microplate washing may be manual, automated or semi-automated, but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned and maintained.

   a. Automatic Microplate Washer – Set the fill volume to 300 µL/well. Prime the instrument with Plate Wash. Use two 3-cycle washes. After the initial 3-cycle wash, invert the plate and firmly strike it against an absorbent surface. Rotate the plate 180 degrees and repeat.

   b. Manual Microplate Washer – Wash six (6) times, using 300 µL/well per wash. Fill the entire plate, then aspirate in the same order.

   c. Hand-held Syringe or Wash Bottle – Wash six (6) times, using 300 µL/well per wash (filling the wells). Blot the plate upside-down between washes.

   After the final wash, invert the microplate and firmly strike it on an absorbent surface. Visually check that all wells are empty.

2. When inverting the microplate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. As an additional precaution, it is advisable to apply tape to the edges of the strips.

3. The Template Solution and reagents containing enzymes (the samples to be tested, any RT standards employed, ELOSA Mix and Probe Solution, 50X) should be kept cold until used.

E. Other Issues

1. DNA-dependent DNA polymerases

Cell extracts may contain DNA-dependent DNA polymerase activities that are distinct from RT, but can generate cDNA from certain RNA homopolymers. The use of a heteropolymeric RNA template in the RT-Detect™ assay prevents these activities from being expressed. Thus, unlike assays which use homopolymer templates, it is *not* necessary with RT-Detect™ to run a parallel reaction using a DNA template to prove that the enzyme activity observed is due to the presence of reverse transcriptase.

2. Proteases

If protease activity in the sample is a concern, 2 mM phenylmethyl sulfonyl fluoride (PMSF) may be added during sample processing with no effect on the RT-Detect™ assay.

F. Troubleshooting

1. The Positive Control DNA from the RT-Detect™ kit can be used as a control for the detection reactions. (Because it is added directly and not generated by reverse transcriptase during the assay, it cannot act as a control for the reverse transcription reactions.) Running a standard curve of the Positive Control DNA as described in the RT-Detect™ kit manual can be useful in trouble-shooting assay problems. Such a standard curves obtained should be nearly linear if the protocol is strictly followed. Variations from the protocol may increase the non-linearity of the standard curve. For a non-linear result, point-to-point or non-linear curve fitting methods should be used.

2. Poor duplicates may indicate inaccurate pipetting or insufficient washing. If all instructions for the preparation and use of Plate Wash were carefully followed, such results may indicate a need for plate washer maintenance.

   Alternatively, poor duplicates may be caused by insufficient mixing after addition of the TMB Stop Solution. This type of problem can be identified by re-reading the absorbance 15 - 60 minutes after addition of the TMB Stop Solution. Insufficiently mixed solutions will equilibrate over this time period and give increased signal and better duplicates.

3. A Substrate Blank Well (a microplate well which is left empty until the TMB Substrate and Stop Solution are added), read against air, should read less than or equal to 0.1 absorbance unit when read at 450 nm with a 650 nm reference filter. A significantly higher value may indicate deterioration of the TMB Substrate solution, but when readings are corrected by subtracting the absorbance of the 0 fmol/mL Positive Control DNA standard or the 0 units/mL RT standard, valid assay results will usually be obtained.

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CYTOMEGALOVIRUS DRUG SUSCEPTIBILITY ASSAY
PLAQUE REDUCTION METHOD

I. PRINCIPLE

Cytomegalovirus (CMV) infections are a major cause of morbidity and mortality among immunocompromised patients. Ganciclovir (DHPG) and Foscarnet (PFA) are antiviral compounds with excellent in vitro activity against CMV. Their use in clinical trials have shown some benefit in treatment of severe CMV infections. The majority of clinical strains of CMV are inhibited by Ganciclovir, showing IC_{50} values below approximately 6 µM, and by Foscarnet, showing IC_{50} values below approximately 400 µM. However, there are recent reports of patients showing progressive CMV disease caused by drug resistant strains of CMV. Whether CMV strains resistant to Ganciclovir and/or Foscarnet will become a major clinical problem as the use of these drugs become more extensive is not known. Therefore, continuous surveillance of sensitivities of CMV isolates to antivirals has become crucial.

The CMV sensitivity assay requires host cells, usually human foreskin fibroblasts (MRHF), infected with the patients virus. The patients CMV strain is tested in parallel with a control strain such as AD-169. The viruses are inoculated into wells containing MRHF cells; after infection the number of plaque forming colonies (PFC) is determined. A predetermined number of PFC are inoculated into the wells of a 24-well plate. After adsorption, the wells are overlaid with agarose containing various concentrations of drug and incubated. When there are an adequate number of plaques in the control wells (wells without drug), the plaques in all wells are stained and counted. The number of PFC in the wells containing drug are compared to those in the control wells and an IC_{50} is calculated.

II. SPECIMEN REQUIREMENTS

CMV isolates, fresh or frozen in LN_2, grown to demonstrate 50-100% CPE.

III. REAGENTS

Trypsin-EDTA--Store at 4-8^{0}C. Observe manufacturer's outdate.

Human Foreskin fibroblasts MRHF (Biowhitaker catalog number 72-213B for 75 mm^2 flask or 72-213F for ampoules of cryopreserved cells). (Procedure for cryopreserved cells can be obtained from Biowhittaker catalog).

Minimum essential medium (Eagle)(MEME)--Store at 4-8^{0}C. Observe manufacturer's outdate.

Fetal Bovine Serum (FBS)--Store frozen at -20^{0}C. Observe manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^{0}C waterbath, then heat-inactivate in a 56^{0}C waterbath for 30 minutes with occasional shaking. The level of water in the waterbath should be as high as the

level of the serum in the bottle. Store at 4-8^{0}C after thawing. Heat-inactivated FBS has a one month outdate.

Phosphate Buffered Saline (PBS) with calcium or magnesium or Hanks Balanced Salt Solution (HBSS)--Store at room temperature. Observe manufacturer's outdate or a one month outdate after opening.

SeaPlaque Agarose--may be obtained from FMC Bioproducts, Rockland, ME). Store at room temperature. Observe manufacturer's outdate.

Ganciclovir (DHPG)--Provided by the Virology Quality Assurance Laboratory (VQA). Store at -70^{0}C or below.

Foscarnet (PFA)--Provided by the VQA. Store at -70^{0}C or below.

Formalin--Store at room temperature. Observe manufacturer's outdate.

Dimethylsulfoxide (DMSO)--Store at room temperature. Observe manufacturer's outdate.

Reagent grade water.

AD169 CMV (clinical cell-associated sensitive strain)--Store in LN_2.

Resistant CMV strain--Store in LN_2.

Ethanol--Store at room temperature. Observe manufacturer's outdate.

Crystal Violet--Store at room temperature. Observe manufacturer's outdate. Prepare 0.8% in 50% ethanol.

Growth Medium (10%). To make 500 mL:

a) 450 mL MEME
b) 50 mL FBS

Growth Medium (2%). To make 500 mL:

a) 490 mL MEME
b) 10 mL FBS

Cryoprotective Medium. To make 10 mL:

a) 7 mL MEME
b) 2 mL FBS
c) 1 mL DMSO

Thaw Medium. To make 10 mL:

a) 9 mL MEME
b) 1 mL FBS

Agarose

a) To prepare a 2X stock (0.8%), weigh 4.0 gm agarose.
b) Add reagent grade water to 500 mL.
c) Autoclave at 15 lbs for 15 minutes at 121^0C.
d) Keep in 56^0C waterbath until used. If prepared in advance, store at 4^0C; boil stock prior to use; let cool in 56^0C waterbath.
e) When ready to overlay plates after virus adsorption, mix 2X concentrations of drug in 2X MEME with 10% FBS and antibiotics with equal volumes of 2X (0.8%) agarose. Final concentration of overlay: 0.4% agarose in MEME with 5% FBS.

Note: Keep MEME at 4^0C until used. Be sure overlay is not too hot before proceeding.

Ganciclovir Concentrations

a) The following <u>final</u> (1X) drug concentrations will be used in the assay:
GCV (µM):

8 concentrations (2-fold dilutions)	0	1.5	3	6	12	24	48	96

b) The drug concentrations are prepared from stock solutions provided by the VQA Laboratory and kept at -70^0C until used. At the time of overlay preparation, thaw drug and warm in water bath, shaking intermittently and/or vortexing or sonicating in a waterbath to <u>ensure complete dissolution</u>.

c) Prepare 2X concentrations of drugs in 2X MEME with 10% FBS and antibiotics. In the final step, add to an equal volume of 0.8% agarose to give final concentration.

d) To overlay 1.5 mL per well in a 24-well plate, 3 wells per drug concentration, prepare 5 mL of each drug concentration in final overlay for each plate inoculated to ensure adequate volume.

<u>For actual volumes used, multiply the volumes given in the table by the number of viruses assayed.</u>

Remember, drug-sensitive and drug-resistant controls must be included in each assay. Therefore at least 3 plates (2 controls, 1 unknown) would be prepared for each assay.

Calculations are based on ganciclovir (GCV) stock concentration of 4.5 mM and serial 2-fold dilutions of drug.

Be sure stock drug is in solution before proceeding.

2X Ganciclovir Concentrations

Initial drug conc	**4.5 mM**	**384 µM**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**0 µM**
Add drug above (volume)	300 µL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
to 2x MEME/ 10% FBS	3216 µL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
Yields(2X) GCV conc	**384 µM**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	
Take (mL) to prepare next dilution	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	N.A.	
Volume remaining	N.A.	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	6 mL	

Final Ganciclovir Concentrations in Agarose Overlay (using 2X from above)

GCV (2X)	**N.A.**	**192 µM**	**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	**0 µM**
Take 2X GCV (mL)		2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Add 0.8% agarose (mL)		2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Yields final (1X) GCV		**96 µM**	**48 µM**	**24 µM**	**12 µM**	**6 µM**	**3 µM**	**1.5µM**	**0 µM**
Overlay (1X) 0.4% agarose/ MEME 5% FBS (mL)		5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

Foscarnet Concentrations

a) The following final (1X) drug concentrations will be used in the assay:
PFA (µM):
8 concentrations 0 25 50 100 200 400 800 1000
(2-fold dilutions except for 1000µM)

b) The drug concentrations are prepared from stock solutions provided by VQA Laboratory and kept at -70^{0}C until used. At the time of overlay preparation, thaw drug and warm in water bath, shaking intermittently and/or vortexing or sonicating in a waterbath to ensure complete dissolution.

c) Prepare 2X concentrations of drugs in 2X MEME with 10% FBS and antibiotics. In the final step, add to an equal volume of 0.8% agarose to give final concentration.

d) To overlay 1.5 mL per well in a 24-well plate, 3 wells per drug concentration, prepare 5 mL of each drug concentration in final overlay for each plate inoculated to ensure adequate volume. For actual volumes used, multiply the volumes given in the table by the number of viruses assayed.

Remember, drug-sensitive and drug-resistant controls must be included in each assay. Therefore at least 3 plates (2 controls, 1 unknown) would be prepared for each assay.

Calculations are based on Foscarnet (PFA) stock concentration of 20 mM and serial 2-fold dilutions except for the initial 1000 µM concentration.

Be sure stock drug is in solution before proceeding.

2X PFA Concentrations

Initial drug conc	**20 mM**	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**0 µM**
Add drug above (volume)	0.8 mL	4.8 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
to 2x MEME/ 10%FBS	7.2 mL	1.2 mL	3 mL	3 mL	3 mL	3 mL	3 mL	
Yields(2X) PFA conc	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	
Take (mL) to prepare next dilution	4.8 mL	3 mL	3 mL	3 mL	3 mL	3 mL	N.A.	
Volume remaining	3.2 mL	3 mL	3 mL	3 mL	3 mL	3 mL	6 mL	

Final PFA Concentrations in Agarose (using 2X from above)

PFA (2X)	**2000 µM**	**1600 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	**0 µM**
Take 2X drug (mL)	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Add 0.8% agarose (mL)	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Yields final (1X) PFA	**1000 µM**	**800 µM**	**400 µM**	**200 µM**	**100 µM**	**50 µM**	**25 µM**	**0 µM**
Overlay (1X) 0.4% agarose/ MEME 5% FBS (mL)	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

IV. EQUIPMENT AND SUPPLIES

Laminar flow (Class 2 biosafety hood)
Gloves
Lab coat
Sterile disposable 1 mL, 2 mL, 5 mL, and 10 mL plugged and unplugged Pasteur pipettes
Sterile 15 mL and 50 mL tubes
25 cm^2 tissue culture flask
24-well tissue culture plates
Sterile cryovials
Hemacytometer
Microscope (inverted, dissecting or phase with low power)
Vacuum aspirator with appropriate trap
Waterbath capable of 56^0C
Incubator at 36-37^0C with 5% CO_2 and humidity
Low speed centrifuge with tube and plate carriers

V. PROCEDURE

A. Propagation and assay of cell-associated virus

1. Obtain CMV clinical isolate, usually a culture in a screw cap tube.

2. Trypsinize cells and transfer cell suspension to a 25 cm^2 flask of human fibroblast cells. When cytopathic effect (CPE) is observed, trypsinize and redistribute cells to provide a more rapid progression of CPE.

3. Pipette medium from the flasks.

4. Rinse monolayer twice with PBS without calcium or magnesium. Add 1-3 mL fresh trypsin to flask. Place at room temperature or at 37^0C for approximately 1-2 minutes or the minimum time needed to loosen the cells.

5. Tap flask sharply to dislodge cell monolayer.

6. Add 5 mL of growth medium, pipette up and down to separate cell clumps, then transfer contents to a fresh 25 cm^2 flask. In some cases, it may be necessary to add fresh fibroblast cells as well.

7. Observe the flask daily and repeat every 3-4 days until the CPE reaches at least 50%.

8. When CPE involves 50-75% of the monolayer, trypsinize cells, gently resuspend in MEME containing 10% FBS and antibiotics; pipette up and down to obtain a single cell suspension.

9. Count cells in a hemacytometer.

Note: Accurate results depend upon having a uniform distribution of a single-cell suspension.

10. Multiply cell count by the estimated percent of viable infected cells to determine the number of plaque-forming cells (PFC) in the suspension.

11. Adjust cell concentration to 400 PFC/mL to provide an inoculum dose of 60-80 PFC/0.2 mL.

12. Prepare in 10% MEME approximately 6 mL of virus stock per panel to be inoculated. Inoculation of panels on the same day is recommended. (Note: 8-10% MEME is used for cell-associated virus stocks and 2% MEME for cell-free virus stocks.)

13. Freeze aliquots of remaining virus stock for retest if needed.

    a. Centrifuge the infected cell suspension at low speed so as not to pack the cells too tightly (i.e., 120-180 x g for 5-10 min).

    b. Resuspend the cell pellet in cryoprotective medium; keep well-mixed since clumping will result in variability in titer between aliquots.

    c. Freeze at a titer of 4×10^5 PFC/mL.

    d. Aliquot into small cryovials (0.5 mL/vial).

e. Freeze slowly and store at -85^{0}C or colder.

f. Thaw quickly prior to use; dilute in MEME with 10% FBS to obtain 400 PFC/mL.

(Note: Freezing of virus stock before assay may contribute in variability in the inoculum dose.)

B. Preparation of MRHF 24-well plates from flasks

Note: The time to dislodge cells from a flask will vary with age of the cells and the activity of the trypsin. The goal is to minimize cell contact with trypsin and to grow healthy cells.

1. Obtain MRHF cells at a population doubling level (PDL) of 23-24. Cells can be used up to PDL 30-35 . Records should be maintained of PDL and cultures labeled accordingly. (To calculate PDL: For cells obtained at PDL 24, after passage at a 1:2 split, the cells would be PDL 25. After passage at a 1:4 split, the cells would be PDL 26.)

2. Aspirate medium from 75 cm^2 flask.

3. Rinse monolayer twice with HBSS or PBS (without calcium or magnesium).

4. Add 3 mL of trypsin to flask.

5. Incubate at room temperature or at 37^{0}C for 1-3 min or the minimum time to loosen the cells.

6. Examine monolayer under the microscope and when cells are rounded, rap flask to dislodge cells.

7. Add 10 mL of 10% MEME and pipette up and down to break up clumps.

8. Add 10% MEME to 75 mL total volume. Pipette to mix well.

9. Seed three 24-well plates with 1.0 mL of cell suspension per well, a 1:2 split.

10. Incubate at 37^{0}C in CO_2 incubator until confluent.

11. Inoculate panels when cells are just confluent, usually 2 days after seeding at a 1:2 split.

12. For each virus isolate, prepare one 24 well panel of MRHF for each drug tested. Note: Subconfluent cells are more sensitive, but may be more fragile. At higher

passage levels, cells may require a longer period to become confluent and are more likely to peel.

C. Plaque reduction assay for drug susceptibility screening of CMV

1. For each virus tested, use one 24-well panel per drug assayed. When assaying cell-associated viruses, do not aspirate the medium from the wells prior to inoculation.

2. Gently pipette virus stock up and down to ensure well-mixed, single cell suspension, then inoculate each well with 0.2 mL of cell suspension containing 60-80 PFC of cell-associated virus.

3. Allow adsorption for 90 min in a 37^0C CO_2 incubator.

4. During the adsorption step, prepare drug concentrations in overlay solutions.

5. After adsorption, carefully aspirate inoculum and medium from the wells. Overlay wells with 1.5 mL 0.4% agarose containing the appropriate concentration of antiviral, 3 wells per drug concentration. Allow overlay to gel at room temperature.

6. Incubate plates at 37^0C in a 5% CO_2 incubator, approximately 7-10 days.

7. Beginning on day 4, examine monolayers in control wells microscopically for plaque formation daily.

8. When plaque formation is well defined and there are an adequate number of plaques in the control wells (40 plaques minimum), fix plates in 10% formalin in PBS. Carefully flip off the agarose layer using a small weighing spatula. Stain the plates with 0.8% crystal violet in 50% ETOH or with methylene blue.

9. Count plaques using an inverted, dissecting, or phase microscope at low power.

VI. RESULTS

A. Calculations

1. Determine mean plaque count for control wells without drug and for each drug concentration.

2. The mean plaque count in control wells with no drug is considered the baseline (100%).

3. For each drug concentration, determine % of plaques remaining compared with control wells without drug.

4. Plot % plaques remaining against drug concentration using semi-log paper.

5. Determine 50% inhibitory concentration (IC_{50}) and 90% inhibitory concentration (IC_{90}).

B. Interpretation

Interpretation of plaque reduction assay results:

Drug	IC50	Interpretation
GCV	$\leq$ 6µM	Sensitive
	>12 µM	Resistant
PFA	< 400 µM	Sensitive

VII. QUALITY CONTROL

To reduce variability, the definition of a plaque should be consistent and one reader should read all the plates.

Note: The area of quality control needs to be further defined in the future. For assay to be acceptable, the following parameters need to be defined:

IC_{50} for control sensitive and resistant viruses must fall within a predetermined acceptable range for that virus strain.

Range of acceptable number of plaques for challenge dose (40-100)

Degree of acceptable secondary plaque formation

Degree of variability in plaque counts between replicate wells

Monolayer condition and degree of monolayer loss

VIII. REFERENCES

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HIV PROVIRAL POLYMERASE CHAIN REACTION (HIV DNA PCR)

I. PRINCIPLE

HIV infection is usually documented by detection of HIV specific antibodies in serum. However, serologic assays do not readily identify HIV infection in neonates with passively acquired maternal antibodies or individuals with "indeterminate" antibody profiles or early infection. These situations have often required follow-up serologic testing, antigen detection and culture.

Gene amplification techniques, e.g., Polymerase Chain Reaction (PCR), allow the detection of rare DNA sequences. PCR can detect low copy numbers (1-5) of HIV-1 proviral DNA in infected cells. PCR may help document HIV infection when routine diagnostic assays are not adequate.

Characteristic of all retrovirus infections, the HIV single stranded RNA is transcribed in double stranded DNA ("provirus") and integrated into the host cell genome. This integrated HIV provirus can be detected by amplification of a specific sequence in the highly conserved gag region of the genome.

Whole blood is treated with a specimen wash reagent which selectively lyses the red blood cells. The intact leukocytes are pelleted and washed. Alternatively, the pellets can be made from the PBMCs isolated using lymphocyte separation techniques described in the Qualitative PBMC Macrococulture Assay Protocol. The pellets can be stored at -70^0C or be treated with nonionic detergents, magnesium and proteinase K to extract the DNA for analysis.

The double stranded DNA is denatured by heat to expose the target region to the labeled primers. The amplification target is a highly conserved region of the gag genome which is bordered by the primer pair SK 431/462. Two biotinylated oligonucleotide primers complementary to the amplification target sequence will bind to the target region on the DNA. The taq polymerase utilizing deoxynucleotide triphosphates, (dATP, dGTP, dCTP, dUTP) extend in the 5' to 3' direction to produce biotinylated complementary DNA sequence called amplicons. During the polymerase chain reaction, controlled fluctuations in temperature allow repeated denaturation, annealing and extension processes resulting in a geometric increase in the target sequences. DNA copies from previous cycles become templates in subsequent amplification periods.

Uracil-N-Glycosylase (UNG) is used to prevent contamination from previous PCR reactions. UNG utilizing strand excision, will excise any dUTP found in previously amplified DNA. Naturally occurring DNA will contain dTTP. UNG is only active at 55^0C. Before the PCR reaction is initiated, the thermocycler is set at 55^0C to activate UNG for two minutes. After the amplification cycles are completed, the UNG is inactivated by a denaturing solution containing sodium hydroxide.

The amplified DNA is incubated onto polystyrene wells which contain immobilized BSA-conjugated probe (SK 102) which is specific to the biotinylated amplicons. After incubating for one hour, the unbound reactants are washed away. An avidin-horseradish peroxidase conjugate is added and incubated for 15 minutes at 37 $\pm$ 1^0C. Unbound reactants are washed away. A chromogentic substrate, tetramethylbenzidine (TMB) is added for a 10 minute incubation. The reaction is stopped by the addition of H_2SO_4 and the absorbance is read at 450 nm. A fixed O.D. cutoff of 0.350 is used to determine whether a specimen is positive or negative.

II. SPECIMEN REQUIREMENTS

Acceptable specimens include whole blood collected in Acid Citrate Dextrose (yellow top) vacutainer or in EDTA (lavender top) vacutainer. Heparinized (green top) blood is not acceptable for this assay. Technically other body fluids may be processed for HIV PCR. However, the HIV PCR assay has been standardized using peripheral blood specimens.

Specimens should be transported and stored at 2-25^0C. Whole blood should be processed to PBMC pellets within 4 days of collection. Specimens should not be frozen prior to separation of PBMCs.

Ill. HIV PCR REAGENTS: Roche - Amplicor Detect™ kits, listed in A, B and C below:

A. Whole Blood Specimen Kit:

1. Specimen Wash Solution (H_2O, Sodium Chloride, Saponin)
2. Extraction reagent (Tween 20, H_2O)

B. HIV-1 Amplification Kit:

1. HIV-1 Positive Control
2. HIV-1 Negative Control
3. H IV-1 Master Mix
4. HIV-1 AmpErase

Positive control: 50 µL positive control + 200 µL extraction buffer
Negative control: 50 µL negative control + 200 µL extraction buffer

C. HIV-1 Detection Kit:

1. Wash Concentrate (H_2O, Sodium Chloride, Tween 20)
2. PCR Denaturation Solution (Sodium Hydroxide, EDTA, H_2O)
3. PCR Hybridization Buffer (Sodium Thiocyanate, Sodium Phosphate, H_2O)
4. PCR Conjugate (TRIS, Avidin-HRPO)
5. PCR Substrate A (H_2O_2, Citric Acid, H_2O)
6. PCR Substrate B (DMF, H_2O, TMB)
7. Stop Reagent (Sulfuric Acid, H_2O)

D. Standards and controls provided by the Virology Quality Assurance Laboratory (VQA)
   1. 0 copy standard
   2. 5 copy standard
   3. 10 copy standard
   4. 20 copy standard
   5. Blinded QC samples

IV. SUPPLIES AND EQUIPMENT

A. Sample Processing / Extraction Room:

40-200 µL pipettor
200-1000 µL pipettor (2)
2 mL sterile Sarstedt tubes
3 mL sterile plastic disposable transfer pipettes
12 x 4 Sarstedt tube rack
2 heating blocks
Table top microcentrifuge

B. Amplification Reaction Room:

0.5-10 µL pipettor
10-50 µL pipettor
40-200 µL pipettor
200-1000 µL pipettor
Eppendorf repeat pipettor
Retainer tray & holder
Aerosol resistant pipettor tips
500 µL snap cap microfuge tubes
1.5 mL snap cap microfuge tubes
1.25 mL sterile Eppendorf repeater tips
Thin-walled reaction tubes & caps

C. Product Room:

0.5-10 µL pipettor
10-50 µL pipettor
40-200 µL pipettor
200-1000 µL pipettor
Multichannel pipettor (5-50 µL)
Multichannel pipettor (50-200 µL)
Aerosol resistant pipettor tips
Retainer tray holder
Retainer tray holder

10 mL pipettes
Sterile reagent boats
Table top microfuge
DNA thermal cycler Perkin-Elmer TC 9600 (Note: if using a different thermocycler adjust program instructions listed below to define appropriate cycle parameters.)

V. PROCEDURE:

Call up an assay template and match specimens with the list. Be sure specimens were stored appropriately, i.e., at 2-8^{0}C.

A. Sample Processing:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves
Location: Sample Preparation Area with a P2 Biological Safety Cabinet

1. For each patient specimen, pipette 1.0 mL specimen wash buffer into a 2.0 mL Sarstedt vial. Note: Each patient sample is run in duplicate.

2. Invert blood specimen in vacutainer tube 15-20 times to mix thoroughly. Add 0.5 mL whole blood to vial containing specimen wash buffer.

3. Seal vial and mix by inversion 10- 15 times.

4. Allow mixture to stand at room temperature for 5 minutes. Vortex specimen vial thoroughly for a minimum of 15-30 seconds. Incubate specimen at room temperature for 5 minutes. Vortex specimen thoroughly for a minimum of 15 - 30 seconds.

5. Centrifuge vial for 3 minutes at 1200 rpm at room temperature in a table top microfuge.

6. Aspirate supernatant being careful to avoid disturbing the pellet. Add 1.0 mL specimen wash buffer to vial. Vortex vial specimen thoroughly for 30 seconds. Centrifuge vial for 3 minutes at 1200 rpm at room temperature.

7. Repeat Step 6 (2nd wash).

8. Aspirate supernatant being careful to avoid disturbing the pellet. The dry pellet may be extracted immediately or stored at -70^{0}C until ready to extract.

NOTE: Pellets may also be prepared from the PBMCs isolated using the lymphocyte separation techniques described in the Qualitative PBMC Macrococulture Assay.

B. Extraction:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves.

Location: Set-Up Area

Check that the 2 separate heating blocks are heated to 60^0C and 100^0C.

1. For each run include a minimum of 3 kit negative controls and 1 kit positive control. Include also one pellet each of VQA 0 Copy, 5 Copy, 10 Copy, 20 Copy Standards and two pellets of Blinded QC Samples.

2. Remove cell pellet from -70^0C. To each pellet, add 200 µL of extraction reagent, vortex for 15-30 seconds. Incubate at 60^0C for 30 minutes.

3. Immediately incubate extract at 100^0C for 30 minutes. Proceed to PCR amplification procedure.

C. Amplification Reactions:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves

<u>Reconstitution of 2x Master Mix-Amperase</u>

Location: "Clean" Pre-PCR "No DNA" BSC Area

1. Generate a plate map from the laboratory computer. Intersperse the Kit Negative controls throughout the patient samples and controls.

2. Add 100 µL of Amperase into one vial of Master Mix. Invert mixture 10-15 times. This mixture is sufficient for 32 amplifications. Write the date of the Master Mix-Amperase preparation onto the vial. This mixture is stable for 4 weeks at 2-8^0C.

3. Place the PCR tubes into the sample retainer tray. Aliquot 50 µL of the 2x master mix into each PCR tube.

Location: "Clean" Pre-PCR "DNA OK" Area

4. Aliquot 50 µL of the extracted sample DNA into the master mix of the appropriate tube. Use positive displacement pipettors and a separate sterile ART pipette tips for each specimen. <u>Use extreme care to avoid carry-over contamination and aerosols</u>. Loosely cap each strip of PCR tubes after inoculation. After all tubes have been inoculated, firmly press caps on using the installation tool.

5. Remove tubes from the retainer tray base. Carry PCR tubes in the white retainer tray directly into the product room. Place retainer tray into the TC 9600 thermal cycler, aligning the notch on the retainer tray with the notch on the TC 9600. Close the cover and turn the cover knob until the white portions of the lid & knob are aligned.

6. a. Begin the HIV-1 proviral PCR program using the following file programs:

Hold:	50^0C,	2 minutes	
Cycle:	95^0C,	10 seconds	
	55^0C,	10 seconds	5 cycles
	72^0C,	10 seconds	
Cycle:	90^0C,	10 seconds	
	60^0C,	10 seconds	30 cycles
	72^0C,	10 seconds	
Hold:	72^0C,	5 minutes	
Hold:	72^0C	"forever"	

The HIV-1 DNA program series requires approximately 75 minutes to complete.

b. To start TC 9600 thermal cycler program:
   1) Turn on switch for TC 9600
   2) "Run" is underlined. Press: enter
   3) Screen: Run Enter Program #
   4) Screen: Reaction volume: "100?"
      Press: enter
   5). Thermal cycler begins. Program file runs for 75 minutes.

(Note: if using a different thermocycler, adjust program instructions listed below to define appropriate cycle parameters as listed above.)

7. As soon as cycles end, press "Stop". TC 9600 temperature ramps down to 25^0C. Allow the TC9600 to hold at 25^0C for 5 minutes to allow condensation to settle.

8. Remove caps very carefully to avoid aerosols. Immediately add 100 µL amplicon denaturation solution to each sample.

9. Let denatured amplicons stand at room temperature for 10 minutes before proceeding to microtiter plate hybridization assay.

D. Detection

Protective Clothing: Disposable isolation gown, shoe covers, 2 pairs disposable gloves.
Location: PCR Product room

MICROTITER PLATE HYBRIDIZATION ASSAY FOR THE DETECTION OF PCR AMPLIFIED HIV-1 SEQUENCES

1. Allow all the reagents to come to room temperature. Remove the appropriate number of microtiter strips from the foil pack and place onto the plate holder. Add 100 µL of hybridization/neutralization buffer to each well.

2. Add 25 µL denatured amplicon to wells containing neutralization/hybridization buffer. Tap plate gently 10-20 times to sufficiently neutralize the amplicons. Neutralization will be evident when the color changes from blue to yellow.

3. Cover microtiter plate with plate cover and incubate for 1 hour at 37^0C.

4. Prepare Working Wash Solution by adding 1 volume of wash concentrate (10x) to 9 volumes of distilled or deionized water. Mix well. After the incubation, wash plate 5 times manually or by using a microwell plate washer.

    a. For Manual washing:

        1) Empty contents of plate and tap dry on paper towels
        2) Pipette working wash solution to fill each well to top (400-450 µL). Let soak for 30 seconds. Empty out contents and tap dry.
        3) Repeat step 2) four additional times.

    b. For Automated washing, program washer to:

        1) Aspirate contents of wells
        2) Fill each well to top with working wash solution (~350-450 µL dependent on plate washer), soak for 30 seconds, then aspirate dry.
        3) Repeat step 2) four additional times. Tap the plate dry.

5. Add 100 µL of Avidin-HRPO conjugate into each well. Cover plate with plate cover and incubate at 37^0C for 15 minutes.

6. Wash microtiter plate as described in step 4.

7. Prepare Working Substrate reagent just prior to use:

    Mix 4 parts PCR chromagen reagent A
    +
    1 part PCR substrate reagent B

Add 100 µL Working Substrate reagent into each well and incubate exactly 10 minutes at room temperature in the dark.

8. Stop reaction with 100 µL stop reagent.

9. Read microtiter plate at 450 nm.

VI. QUALITY CONTROL

The controls must react as expected:

- Kit Positive control should have the A_{450} reading 2.0 or greater (preferably 3.0).
- Kit Negative control must be negative. A_{450} reading should be 0.250 or less.
- VQA 5,10, and 20 copy standards must be positive.
- VQA 0 copy standard must be negative.
- Blinded pellets will be evaluated by the VQA when the data is exported.

A run with one or more controls out of line will usually need to be repeated.

VI. REPORTING RESULTS

1. Absorbances 0.350 or greater are considered positive.

2. Absorbances below 0.350 are considered negative.

All specimens are run in duplicate. If these duplicates have discordant results, the assay will need to be reviewed and the specimens may require retesting.

VII. PROCEDURE NOTES

1. To avoid contamination of specimens and reagents:

   a. No aliquot is ever returned to the original container.

   b Only one tube or container is opened at a time.

   c. Use aerosol resistant tips for all pipettors.

   d. Use different tips or pipettes for each reagent or specimen.

   e. Do not insert pipettor beyond the disposable tip into any container.

2. The submitting physician or laboratory must be called if a specimen is deemed incorrect, inadequate or if there is a failed run.

3. At each step, specimen labeling and handling must be optimized to prevent mix up or contamination.

If any negative controls are positive, suspect contamination. Clean up the area and equipment with a solution of 1M NaOH or 1M HCl to remove any contamination with PCR product. Repeat the assay to verify results.

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CHIRON QUANTIPLEX® HIV RNA 2.0 ASSAY
HIV-RNA Quantitation by Branched DNA (bDNA)

I. PRINCIPLE

The Quantiplex® HIV-RNA Assay is a sandwich nucleic acid hybridization procedure for the quantification of HIV type 1 RNA in human plasma. HIV is first concentrated from plasma via centrifugation. After HIV genomic RNA is liberated from virions, its capture to a microwell is mediated by a set of specific, synthetic oligonucleotide target probes. Another set of target probes hybridize to the viral RNA and the pre-amplifier probes. The pre-amplifier probes hybridize to the branched DNA (bDNA) amplifiers. Specifically, the two sets of target probes bind to different regions of the pol gene of the viral RNA.

Multiple copies of an alkaline phosphatase-labeled probe are hybridized to the immobilized complex to amplify the signal. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission generated by the bound alkaline phosphatase. Light emission is directly proportional to the amount of HIV RNA present in each sample, and results are recorded as luminescent counts by a plate luminometer. A standard curve is defined by light emission from standards with known concentrations of recombinant bacteriophage. Concentrations of HIV-RNA in specimens are determined from the curve.

II. SPECIMEN REQUIREMENTS

The bDNA assay has been optimized for 1.0 mL plasma collected in EDTA. Acid Citrate Dextrose (ACD) is also an acceptable anticoagulant. Heparin may result in depressed quantitation and should not be used.

No special preparation of the patient is required prior to specimen collection. However, proper specimen handling is extremely important.

Store whole blood at room temperature before separating plasma from cells; **Do Not refrigerate.**

Use standard procedures to remove plasma from cells within 4 to 6 hours of collection. Do not clarify plasma by filtration. If the plasma specimen is not to be tested within 30 minutes of separation, store it at -60^{0}C to -80^{0}C in a sterile, screw-capped tube. It is convenient to freeze specimens in accurately measured 1 mL aliquots. Avoid repeated thawing and freezing.

III. REAGENTS

Chiron Quantiplex® HIV RNA 2.0 Assay Kit:

1. Box 1 (shipped refrigerated at 2-8^{0}C) Component:

a. HIV Wash A (buffered solution with sodium azide and antimicrobial preservatives)

b. HIV Wash D (buffered solution with sodium azide and antimicrobial preservatives)

c. Plate Sealers

d. HIV Capture Wells (polystyrene microwell strips coated with synthetic oligonucleotides)

e. HIV lysis diluent (buffered solution containing protein and synthetic oligonucleotides with sodium azide and antimicrobial preservatives)

f. HIV lysis reagent (stabilized proteinase K solution)

g. HIV label diluent (buffered solution with protein stabilizers; with sodium azide and antimicrobial preservatives)

h. HIV substrate (chemiluminescent substrate - Lumi-Phos® Plus)

i. HIV Substrate Enhancer (contains 0.05% sodium azide and 0.05% Proclin™ 300 as preservatives)

j. HIV Pre-Amplifier (buffered solution containing protein™ and synthetic oligonucleotides with sodium azide and Proclin 300 as preservatives)

k. HIV Amplifier (buffered solution with protein stabilizers and synthetic oligonucleotide molecules including branched DNA with sodium azide and antimicrobial preservatives)

l. HIV Standards Diluent (buffered solution containing protein and synthetic oligonucleotides with sodium azide and antimicrobial preservatives)

m. HIV Bead Suspension (buffered solution containing inert polystyrene beads with 0.05% sodium azide and Proclin™ 300 as preservative)

n. HIV Target Probes (synthetic oligonucleotides in water with sodium azide and antimicrobial preservatives)

2. Box 2 (shipped frozen on dry ice) Component:

a. HIV Label Probe (enzyme-labeled synthetic oligonucleotide in buffer solution with sodium azide and antimicrobial preservatives)

b. HIV Positive Control (human plasma containing beta propriolactone-treated HIV with sodium azide and antimicrobial preservatives)

c. HIV Negative Control (human plasma nonreactive for HIV-RNA and nonreactive for HIV antibody, with sodium azide and antimicrobial preservatives)

d. HIV Standard A (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

e. HIV Standard B (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

f. HIV Standard C (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

g. HIV Standard D (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

3. VQA Control provided by the Virology Quality Assurance Laboratory (VQA)

Storage of reagents:

Observe manufacturer's outdates.

Box 1 is shipped refrigerated and should be stored refrigerated (2-8^{0}C) upon receipt.

Box 2 is shipped frozen on dry ice. Store Box 2 and its entire contents frozen (-60 to -80^{0}C)

VQA reagents should be stored in accordance with VQA instructions

IV. EQUIPMENT AND SUPPLIES

Laminar flow biosafety cabinet (class II)
Gloves
Lab coat
Adjustable micropipettors (1000 µL, 200 µL, 20 µL)
Sterile, disposable tips, with and without aerosol resistant barriers

Multichannel pipette, 12-channel, with 15 µL, 150 µL, and 200 µL volumes
Disposable reagent reservoirs for multichannel pipette
Vortex mixer
Refrigerated centrifuge (RCF = 23,500 x g), 24 tube fixed angle rotor (45)
Dry heat block (53^0C)
Incubator or waterbath (37^0C)
Eight welled aspirator, connected to 4L vacuum flask and vacuum pump
Chiron® heater (53^0C) with 8 x 12 metal microwell holder
Chiron® luminometer (37^0C ± 2.5^0C)
Screw-cap, conical, sterile, 1.5 mL microcentrifuge tubes with o-rings
Sterile, disposable plastic 15 mL and 50 mL tubes
Sterile, disposable plastic 6 mL tubes
Blank microwells
Sterile, serological pipettes
Household bleach (5.25% sodium hypochlorite or equivalent)

V. PROCEDURE

The Quantiplex® HIV-RNA Assay procedure consists of two parts: (1) specimen preparation and (2) the assay procedure itself. Specimens are prepared by centrifugation, which concentrates the virus into a pellet. Pellets can be prepared in advance and stored for up to 2 weeks at -60^0C to minus 80^0C. It is convenient and cost effective to prepare specimens in advance and test them in a full or half plate, batch mode. If pelleted specimens are not to be tested immediately *(*within 10 minutes*)* after preparation, they must be frozen.

Each kit contains sufficient reagents and materials for one 96-well run, consisting of 4 standards, 2 controls, and 84 patient specimens, to be tested. When assaying partial plate, prepare only half volumes of working reagents, and store unused reagents and kit components as directed. Fill unused plate positions with blank microwells.

Using fewer than 48 wells at one time is not recommended.

A. Critical parameters

1. Bring assay components to indicated temperature before use.

2. Thaw frozen reagents immediately before use by placing in cool tap water. Vortex briefly.

3. Prepare working reagents immediately before use as instructed in the assay procedure.

4. Return all assay components to recommended storage conditions after use.

5. Add all reagents and specimens to HIV capture wells by touching the pipette tip to the wall near the midpoint of the well, above the surface of the fluid in the well.

6. For best results, do not break apart HIV capture well strips into small segments, as they may adhere to the plate sealer and spill their contents upon its removal.

7. HIV capture wells must be sealed securely during incubations to prevent evaporation. Before each incubation, press a new plate sealer very firmly onto the tops of the wells, using a sweeping motion of the palm of the hand or a flat object.

8. When removing the plate sealer following incubations, use care not to pull the HIV capture wells out of the plate holder. Use one hand to hold the wells in the plate holder while slowly and carefully peeling back the plate sealer with the other hand.

9. When vortexing materials, mix with at least five complete pulses.

10. Assay all Standards, Negative control, and Positive control in duplicate.

11. Before adding materials to HIV capture wells, position the metal plate holder with its notches on the left.

B. Preparation of specimens and controls

1. Pre-chill the centrifuge and rotor to 4^0C.

2. In a Biological Safety Class II cabinet, prepare a vacuum flask containing 100 mL of household bleach and a secondary trap attached to a vacuum source. Connect a plastic aspirating device to the vacuum flask.

3. Thaw one rotor's complement of specimens and the HIV Positive, Negative and VQA Controls in cool tap water for approximately 10 minutes. As soon as the specimens and controls are thawed, proceed to the next step. Do not leave the thawed specimens in tap water for an extended period of time.

4. Transfer 1 mL of specimen or control aliquots into labeled 1.5 mL screw-capped tubes.

5. Mix HIV Bead Suspension by inversion. Add 50 µL HIV Bead Suspension to each 1 mL specimen or control.

6. Cap tubes and centrifuge at 23,500 x g for 1 hour at 2 to 8^0C.

7. In a biological safety cabinet, open the rotor and carefully remove tubes.

8. Immediately aspirate the supernatant from each control and specimen using a plastic aspirating device equipped with a clean, sterile, disposable 200 µL plastic tip. Use a new tip for each specimen and control. Do not disturb the pellet, the location of which is indicated by the colored microparticles. Do not aspirate the pellet to dryness; leave 15 to 20 µL of supernatant.

9. Immediately (within 10 minutes) proceed with the assay procedure or freeze the virus pellets at -60^{0}C to -80^{0}C. Pelleted virus is stable for up to 2 weeks when frozen.

C. Assay procedure--day 1

1. Preheat the Chiron® Heater to 53 ± 0.5^{0}C for at least 30 minutes. (The Chiron® Heater may be left on for an extended period of time between assay runs.)

2. Remove HIV Lysis Diluent, HIV Standards Diluent, HIV Lysis Reagent, HIV Target Probes, HIV Capture Wells, HIV Wash A and D, and Plate Sealers from BOX 1. Warm HIV Lysis Diluent and HIV Standards Diluent to 37^{0}C for 10 to 15 minutes before use. Mix HIV Lysis Diluent and Standards Diluent by inversion.

3. Prepare HIV Specimen Working Reagent by pipetting 20 mL of HIV Lysis Diluent into a sterile 50 mL tube and adding 140 µL of HIV Target Probes and 2.4 mL of HIV Lysis Reagent (full plate). For half plates, add 10 mL of lysis diluent, 1.2 mL of lysis reagent, and 70 µL of HIV target probe into the sterile tube. Mix by inversion and maintain at room temperature (15^{0}C-30^{0}C) for up to 4 hours. See figure 3A, 3B, 3C and 3D for Reagent Preparation Chart.

4. Prepare HIV Standards Working Reagent by pipetting 1.5 mL of HIV Standards Diluent into a sterile 6 mL tube and adding 15 µL of HIV Target Probes and 270 µL of HIV Lysis Reagent. Mix by inversion and maintain at room temperature (15^{0}C-30^{0}C) for up to 4 hours.

5. Remove one rotor's complement of pelleted specimens or controls (in groups of 24, staggered 10 minutes apart) from the freezer and add 220 µL of Specimen Working Reagent to each tube. Cap the tubes and vortex for 10 seconds. **NOTE: If pellets were frozen, thaw at room temperature up to 5 minutes prior to adding HIV Specimen Working Reagent.**

6. Incubate tubes in a heat block at 53^{0}C for 20 ± 2 minutes.

7. During the incubation, set up the microwell plate as shown in Figure 1 or Figure 2.

Figure 1
Microwell Full Plate Setup

STD A	**STD A**	**STD B**	**STD B**	**STD C**	**STD C**	**STD D**	**STD D**	**NEG**	**NEG**	**POS**	**POS**

VQA	VQA	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC

Figure 2
Half Plate Setup

STD A	**STD A**	**STD B**	**STD B**	**STD C**	**STD C**	**STD D**	**STD D**	**NEG**	**NEG**	**POS**	**POS**
VQA	VQA	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC

a. Allow the HIV Capture Well pouch to reach room temperature before opening. Remove HIV Capture wells from their pouch and place them in the metal 8x12 microwell holder provided with the Chiron Heater. Break the plastic tabs at the end of each strip. (If only 48 wells are needed, return the remaining 48 wells to the pouch, seal with tape, and store at 2^0C- 8^0C. Fill the lower half of the plate holder with blank microwells. Do not distribute reagents to blank wells.)

b. Record positions of HIV Standards, HIV Controls, VQA Control and patient specimens on Plate Map.

Figure 3A
HIV Specimen Working Reagent Preparation

Number of HIV Capture Well Strip	**HIV Lysis Diluent**	**HIV Lysis Reagent**	**HIV Target Probes**
4 (1/2 plate)	10 mL	1.2 mL	70 µL
8 (full plate)	20 mL	2.4 mL	140 µL

Figure 3B
HIV Standards Working Reagent Preparation

Number of HIV Capture Well Strip	HIV Lysis Diluent	HIV Lysis Reagent	HIV Target Probes
4 (1/2 plate) or 8 (full)	1.5 mL	270 µL	15 µL

Figure 3C
HIV Label Working Reagent Preparation

Number of HIV Capture Well Strip	HIV Label Diluent	HIV Label Probe
4 (1/2 plate)	3 mL	50 µL
8 (full plate)	6 mL	100 µL

Figure 3D
HIV Substrate Working Reagent

Number of HIV Capture Well Strip	HIV Substrate	HIV Substrate Enhancer
4 (1/2 plate)	1.5 mL	1.5 mL
8 (full plate)	3 mL	3 mL

8. Following the incubation, remove the tubes from the heat block and vortex for 10 seconds.

9. Hold tubes at room temperature for 5 minutes.

10. During the final heat block incubation, thaw HIV Standards A, B, C, and D at room temperature.

11. Transfer 200 µL of patient specimens, and controls into the appropriate HIV Capture Wells.

12. After all specimens and controls are loaded into the HIV Capture Wells, add 150 µL of HIV Standards Working Reagent to those wells designated for HIV Kit Standards.

13. Vortex Kit HIV Standards A, B, C, and D and add 50 µL of each Standard to the appropriate wells.

14. Seal the plate with a plate sealer, pressing firmly to avoid vapor leaks. Ensure that every microwell if fully covered.

15. Immediately incubate the plate in the Chiron® Heater at 53 ± 0.5^{0}C for 16 to 18 hours. Refer to the Chiron® Heater Operator's Manual for proper setup and use.

    Note: Immediately vortex pellets and incubate at 53^{0}C after addition of Specimen Working Reagent.

D. Assay procedure--day 2

16. Warm HIV Wash A and HIV Wash D to room temperature (15^{0}C-30^{0}C).

17. Warm HIV Pre-Amplifier, HIV Amplifier and HIV Label Diluent at 37^{0}C for at least 10 to 15 minutes until solutions are homogeneous. Mix by inversion and use within 2 hours.

    Note: If solutions are not homogeneous, continue heating until reagent is in solution.

18. Prepare a 4 L vacuum flask with 100 mL of household bleach. Connect the multiwell aspirator to the vacuum flask.

19. Carefully remove the plate from the heater. After an overnight incubation, the HIV Capture Wells may stick in the lid of the Chiron® Heater. If this occurs, use a spatula to dislodge the plate and remove carefully. Set the plate on an uncovered bench top to cool for 10 ± 1 minutes. Carefully observe wells and note any well from which fluid has evaporated.

20. Carefully peel back the Plate Sealer with one hand, using the other hand to hold the HIV Capture Wells in place. Discard Plate Sealer.

21. Using an 8-channel microwell vacuum manifold or equivalent, aspirate the entire contents of each well into the vacuum flask containing the disinfectant solution.

22. Using the multichannel pipette or bottle top dispenser, fill each HIV Capture Well with approximately 400 µL of HIV Wash A. Then, completely remove HIV Wash A from every well by aspirating contents into the vacuum flask. Repeat this wash cycle a second time; be certain to thoroughly aspirate the second wash.

    Note: Do not allow HIV Capture Wells to remain dry for more than 5 minutes.

23. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Pre-Amplifier to each HIV Capture Well. Deliver the solution to the side of the well. Cover the plate with a plate sealer as previously described.

24. Incubate the plate in the Chiron® Heater at 53^{0}C for 30 ± 3 minutes.

25. Carefully remove the plate from the heater. Set the plate on a bare bench top or other cool surface at room temperature for 10 ±1 minutes. Observe and record any well from which fluid has evaporated.

26. Carefully remove the plate sealer.

27. Repeat step 21 and 22 as previously described.

28. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Amplifier to each HIV capture well. Deliver the solution to the side of the well. Seal the plate with a fresh plate sealer as previously described.

29. Incubate the plate in the Chiron® Heater at 53 ± 0.5 C for 30 ± 3 minutes.

30. During the incubation, thaw HIV Label Probe at room temperature and vortex to mix.

31. Repeat step 25 as previously described.

32. Prepare HIV Label Working Reagent by pipetting 6 mL of HIV Label Diluent into a sterile 15 mL tube and adding 100 µL of HIV Label Probe. Mix by inversion and maintain at room temperature. Use within 10 minutes. Return remaining Label Probe to -60 to -80^{0}C.

33. Carefully remove the plate sealer and discard. Thoroughly aspirate the contents of all wells, and wash wells twice with HIV Wash A as described in step 21 and 22.

34. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Label Working Reagent to each HIV Capture Well. Securely seal the plate with a new Plate Sealer as described previously. Incubate in Chiron® Heater at 53^{0}C for 15 ± 2 minutes.

35. During this incubation period, program the Chiron® Luminometer to read the plate as instructed in the Chiron® Luminometer Operator's Manual. Users of the Chiron® Date Management System should prepare it to receive data according to the software instructions.

36. During the label incubation, bring the HIV Substrate and HIV Substrate Enhancer to room temperature.

37. Remove the plate from the Chiron® Heater and place it on an uncovered bench top at room temperature for 10 ± 1 minutes. Carefully remove and discard Plate Sealer. Thoroughly aspirate contents from wells, and wash wells twice with HIV Wash A as described previously.

38. Aspirate the wells thoroughly. Use the multichannel pipette or a second bottle top washer to wash wells 3 times with HIV Wash D in the same manner described previously. Be certain to remove all of HIV Wash D after the final wash step.

39. Prepare HIV Substrate Working Reagent by pipetting 3 mL of HIV Substrate into a sterile-packaged polypropylene tube and adding 3 mL of HIV Substrate Enhancer. Mix by inversion. Use within 10 minutes. Solution will be cloudy.

40. Add 50 µL of HIV Substrate Working Reagent to each well using the multi-channel pipette. Securely seal the plate with a Plate Sealer.

41. Immediately insert the plate into the Chiron® Luminometer, aligning the notches in the plate with the pins in the loading tray. Incubate the plate for 30 minutes at 37 ± 2.5^{0}C. The Luminometer will automatically read the relative light units in each of the wells at the end of the 30 minute incubation period.

    Note: When processing more than one plate, begin incubating the second plate 5 minutes after the first. Incubate the second plate in the 37^{0}C dry incubator for 25 minutes and transfer it to the Chiron® Luminometer to incubate at 37^{0}C for the final 5 minutes prior to reading.

42. When the plate reading is completed, proceed as instructed in the Chiron® Data Management Software Manual.

VI. CALCULATION AND INTERPRETATION OF RESULTS

The Chiron® luminometer reports results in relative luminescence (RL) units, which are a measure of the amount of light emitted from each microwell. Light emission is directly proportional to the number of HIV-RNA copies present in each patient specimen.

To quantitate HIV-RNA, the mean RL value of each specimen is compared to the standard curve. The final result is reported in HIV-RNA copies x 10^3/mL.

The Chiron® Data Management System software automatically calculates average relative luminescence values and %CV (coefficient of variation) for HIV Standards, HIV Controls, and patient specimens, plots the standard curve, calculates the HIV-RNA copies x10^3/mL for each patient specimen, and prints out a report.

A. Interpretation of Results

1. The cutoff value for the Quantiplex® HIV-RNA Assay is 0.5 RNA copies x 10^3/mL.

2. Specimens with values above or equal to (≥) 0.5 RNA copies x 10^3/mL contain HIV-RNA in the quantity indicated.

3. Specimen with values less than (<) 0.5 RNA copies x 10^3/mL are below the detection limit of the assay.

4. Specimen with values greater than (>) the assigned value of HIV Standard A are above the upper limit of the Standard Curve and must be diluted to obtain a quantitative value.

VII. QUALITY CONTROL AND EXPECTED VALUES

1. The assay control and standards supplied with the test kit must be included with each run, regardless of the number of specimens tested or capture wells used. HIV value assignments and the expected quantitation of the Positive control are shown in the Product Insert Supplement provided with each kit.

2. If the quantification of the HIV Positive Control is outside of the range listed in the supplement, the entire run is invalid and must be repeated.

3. The negative control must be below the cutoff, if it quantifies, the run is invalid and must be repeated.

4. If the relative luminescence (RL) units for the kit standards do not meet the following criteria: "RLU STD A > RLU STD B > RLU STD C > RLU STD D", the run is invalid and must be repeated.

5. Similarly, VQA standards must yield expected results or the entire assay may need to be repeated.

VII. REFERENCES

1. Chiron® Product insert (Quantiplex® HIV-RNA), L6170 rev 5.0, June 1994

2. Personal communications: Chiron® Technical Services

ROCHE AMPLICOR HIV MONITOR™ ASSAY
Quantitative plasma HIV-1 RNA RT/PCR Assay

I. PRINCIPLE

The Amplicor HIV Monitor™ Test is a PCR system for the quantitative measurement of HIV viral RNA in plasma. The plasma is extracted with a Lysis Reagent containing guanidine thiocyanate and Quantitation Standard (QS) RNA. A known amount of QS is introduced into each sample with the Lysis Reagent to permit quantitation of HIV RNA from a comparison of resulting optical densities following amplification and detection. The RNA is precipitated with isopropanol and resuspended in a buffer containing carrier RNA. A 142 base pair sequence in the gag gene of HIV (primers SK431 and SK462) is amplified by reverse transcription (RT) and Polymerase Chain Reaction (PCR) in a single reaction. The primers are biotinylated at the 5' ends to yield biotinylated amplification products, or amplicons. The biotinylated HIV and QS amplicons are detected in separate wells of a microwell plate (MWP) coated with HIV-specific and QS-specific oligonucleotide probes, respectively. To measure the HIV and QS amplicons over a large dynamic range, 5-fold serial dilutions of the amplicons are made in the HIV-specific and QS-specific wells of the MWP. The bound, biotinylated amplicons are quantified with an Avidin-horseradish peroxidase (HRP) conjugate and a colorimetric reaction for HRP. The HIV RNA copy number is then calculated from the known input copy number of the QS RNA, the optical densities (450 nm) of the HIV well and the QS well that fall within a defined range, and the dilution factors associated with the selected wells.

II. SPECIMEN REQUIREMENTS

Plasma collected in an ACD or EDTA tube and removed from the cells within 6 hours of collection is required for the Roche Amplicor HIV Monitor Assay. The plasma must be stored at -20^0C or below until testing. Freeze/thawing should be avoided. Plasma specimens may be frozen and thawed up to 3 times. Heparinized plasma cannot be used.

III. REAGENTS

VQA standards at 0, 1.5×10^4, 1.5×10^5 and 7.5×10^5 HIV RNA copies/mL. Store at -70^0C.

Roche AMPLICOR HIV MONITOR™ TEST kits. Stored in accordance with manufacturer's specifications.

Isopropanol (reagent grade).

Ethanol, 70% (v/v) prepared from absolute or reagent grade stocks using deionized water.

Distilled or deionized water.

IV. Equipment and Supplies

Area 1: Reagent Preparation

Gloves
Laboratory coat
MicroAmp® Reaction Tubes and caps
MicroAmp® tray, retainers and base (for Perkin-Elmer GeneAmp®
PCR System 9600 or System 2400 thermal cycler)
Micropipettes (adjustable volume, 20-20µL) with plugged (aerosol barrier) tips
Repeat pipettor and individually wrapped tips
Plastic resealable bags

Area 2: Specimen Preparation

Gloves
Laboratory coat
Vortex mixer
Microcentrifuge (minimum RCF 15,500 x g, fixed angle rotor preferred)
Pipettors, 200 µL to 1000 µL volume capacity with aerosol barrier tips
2.0 mL polypropylene, screw cap microcentrifuge tubes
Sterile-filtered deionized water
Sterile disposable polystyrene pipettes (5, 10, 25 mL)

Area 3: Amplification and Detection

Gloves
Laboratory coat
Multichannel pipette or PCR AMPLICOR^{TM} Electronic Impact Pipettor
Pipettors, 50 µL to 1000 µL volume capability
Plugged (aerosol barrier) micropipette tips* (200 µL) and unplugged tips (200 µL)
Perkin-Elmer GeneAmp® PCR System 9600 or System 2400 thermal cycler
Microwell plate washer.§
Microwell plate reader†.
Disposable reagent reservoirs
Microwell plate lid
Incubator $37^0C \pm 2^0C$
Graduated vessels

* Pipettes should be accurate within 3% of stated volume. Plugged (aerosol barrier) tips must be used to prevent sample and amplicon cross contamination.

§ Capable of washing 12 x 8 microwell format with 350-450 µL of Wash Solution per well at 30 second time intervals.

† Microwell reader specifications: bandwidth=10±3 nm, absorbance range=0 to 3.00 A_{450}, repeatability 1%, accuracy 3% from 0 to 2.00 A_{450}, drift 0.01 A_{450} per hour.

V. PROCEDURE

This procedure should be performed in three areas of the laboratory as directed in the following instructions. All reagents must be at ambient temperature before use. Use micropipettors with plugged (aerosol barrier) tips.

A. Master Mix Preparation
Performed in Reagent Preparation Area (Area 1)

1. Prepare Working Master Mix by adding 100 µL of Manganese Solution to one tube of HIV Master Mix. Recap the Master Mix tube and mix well by vortexing 3-5 seconds or by inverting the tube 10-15 times. The pink dye indicates that the manganese has been added to the Master Mix. Master Mix and Manganese are provided in single-use vials, sufficient for 12 tests. Unused Working Master Mix must be discarded.

2. Determine the number of MicroAmp® Reaction Tubes required. The recommended batch size is 12 tests, although a total of 24 tests can be done simultaneously. Place Reaction tubes in MicroAmp® tray, lock tubes in position with tube retainer, and place in base. Place 12 tubes for each batch in a single row.

3. Pipette 50 µL of Working Master Mix into each tube using a micropipettor with a plugged tip or repeat pipettor. Check for pink color to confirm that manganese was added to the Master Mix. Discard unused Working Master Mix.

4. Place the MicroAmp® tray in a plastic, resealable bag, and move to the Specimen Preparation Area (Area 2). Store MicroAmp® tray at 2-8^{0}C until specimen preparation is completed. Amplification must begin within 4 hours of preparation of Working Master Mix.

B. Specimen preparation
Performed in Specimen Preparation Area (Area 2)

1. Prepare 70% ethanol. For 12 tests, mix 11.0 mL 95% ethanol and 4.0 mL of deionized water. This solution should be made fresh on each sample processing day.

2. Prepare the Working Lysis Reagent by adding 100 µL of the Quantitation Standard (QS) to the entire vial of Lysis Reagent. Be sure all crystals in the Lysis Reagent are dissolved before adding QS.

3. Label one 2.0 mL screw cap microcentrifuge tube for each sample and control.

4. Thaw plasma specimens at room temperature and vortex 3-5 seconds.

5. Dispense 600 µL Working Lysis Reagent into each tube.

6. Add 200 µL sample or control to each labeled tube containing Working Lysis Reagent. Immediately cap and vortex 3-5 seconds. When Roche Controls are used:

    a. Add 200 µL of each patient specimen to appropriate tube.

    b. For each Negative and Positive Control, add 200 µL HIV Negative Human Plasma to the appropriate tubes. Cap the tubes and vortex for 3-5 seconds.

    c. Add 50 µL HIV-1 Monitor Negative Control, Low Positive Control and High Positive Control to the appropriate tubes. Cap the tubes and vortex.

7. Incubate tubes for 10 minutes at room temperature.

8. Remove cap and add 800 µL 100% isopropanol to each tube. Vortex for 3-5 seconds.

9. Put an orientation mark on each tube and place tubes into the microcentrifuge with the mark facing outward so that after centrifugation, the pellet will align with the orientation mark. Centrifuge samples at 12,500 x g for 15 minutes at room temperature.

10. Beginning with the control tubes, carefully draw off the supernatant without disturbing the pellet (which may not be visible) using a fine tip, disposable transfer pipette. Slide the pipette down the outside of the tube along the side opposite the pellet while drawing off the liquid. Maintain a continuous negative pressure with the pipette as you draw off the liquid. It is important to remove as much liquid as possible without disturbing the pellet.

11. Add 1.0 mL 70% ethanol to each tube, re-cap, and vortex 3-5 seconds.

12. Place tubes into the microcentrifuge with the orientation mark again facing to the outside so that the pellet will align with the orientation mark. Centrifuge samples at 12,500 x g for 5 minutes at room temperature.

13. Beginning with the control tubes, carefully remove the supernatant as before without disturbing the pellet. The pellet should be mostly white and clearly visible at this step. Remove as much of the supernatant as possible. NOTE: Residual ethanol can inhibit the amplification.

14. Add 400 µL Specimen Diluent.

15. Vortex vigorously for 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

16. Amplify the processed specimens within 2 hours or store at -20^{0}C or colder. Pipette 50 µL of prepared controls and patient specimens into the appropriate MicroAmp® tubes containing previously pipetted Working Master Mix, using a micropipettor with plugged tips. Cap the tubes. Apply pressure for a tight seal using the MicroAmp® Cap Installing Tool.

17. Move the tray of capped tubes to the Amplification and Detection Area (Area 3).

C. Amplification

Performed in Amplification and Detection Area (Area 3)
Note: Thermal cycler must be turned on at least 30 minutes prior to amplification.

1. Place the MicroAmp sample tray (without base) into the thermal cycler sample block.

2. Program the Perkin-Elmer GeneAmp® PCR System 9600 or System 2400 for the Amplicor HIV Monitor™ Test as follows:

Hold	2 min. 50^{0}C
Hold	30 min. 60^{0}C
4 cycles	10 sec. 95^{0}C, 10 sec. 55^{0}C, 10 sec. 72^{0}C
26 cycles	10 sec. 90^{0}C, 10 sec. 60^{0}C, 10 sec. 72^{0}C
Hold	15 min. 72^{0}C (not to exceed 15 mins.)

In the Cycle programs, the ramp time and allowed set point error should be left at the default settings of 0:00 (which is the maximum rate) and 2^{0}C, respectively. Link the 5 programs together into a Method program.

3. Start the Method program (the program runs approximately one hour and 30 minutes).

4. Remove the sample tray from the thermal cycler at any time during the final Hold program. Do not allow the tubes to remain in the thermal cycler beyond the end of the final Hold program, and do not extend the final Hold program beyond 15 minutes. **Do not bring amplified DNA into Area 1 or Area 2. The amplified controls and specimens should be considered a significant potential source of DNA contamination.**

5. Carefully remove caps to avoid creating aerosols. Immediately pipette 100 µL of Denaturation Solution into each PCR tube using a multichannel pipettor, and mix by pipetting up and down 5 times. (Program 1, AMPLICOR™ Electronic Impact Pipettor; see instructions). The denatured amplification reaction mixtures should be held at room temperature no more than 2 hours before proceeding to detection. If detection will not be performed within 2 hours, re-cap the tubes and store the denatured amplification mixtures at 2-8^{0}C for up to one week.

D. Detection

Performed in Amplification and Detection Area (Area 3)

1. Warm all reagents to room temperature prior to use.

2. Prepare Working Wash Solution as follows. Examine the Wash Concentrate for precipitation and, if necessary, warm at 30^{0}C to redissolve any precipitate. Add 1 volume of 10X-Wash Concentrate to 9 volumes of distilled or deionized water. Mix well. The volume of Working Wash Solution required depends on model of washer being used. Store Working Wash Solution for up to 2 weeks at 4-25^{0}C in a clean, closed plastic container.

3. Allow Amplicor HIV Monitor microwell plate (MWP) to warm to room temperature before removing from the foil pouch. Add 100 µL of Amplicor HIV Monitor Hybridization Buffer to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor; see instructions). Rows A-F of the Amplicor HIV Monitor MWP are coated with the HIV-specific oligonucleotide probe; rows G and H are coated with the QS-specific oligonucleotide probe.

4. Using a 12-channel pipettor with plugged tips, add 25 µL of each denatured PCR reaction mixture to a separate HIV well in row A of the MWP and mix by pipetting up and down 10 times. Make serial 5-fold dilutions in the HIV wells (rows B-F), as follows. Transfer 25 µL of the mixture from row A to row B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25 µL. Discard pipette tips. (Addition of denatured PCR reactions to MWP and the serial dilutions may be done with Program 3 of the AMPLICOR™

Electronic Impact Pipettor. This program transfers 25 µL, mixes by pipetting 60µL 5 times, and aspirates 25 µL; see instructions).

5. Again, using a 12-channel pipettor with plugged tips, add 25 µL of each denatured PCR reaction mixture to a separate QS well in row G of the MWP and mix by pipetting up and down 10 times. Make one 5-fold dilution in the QS wells of row H, as follows. Transfer 25 µL from row G to row H, mix as before, remove 25 µL from row H and discard. (Program 3 of the AMPLICOR™ Electronic Impact Pipettor may be used.)

6. Cover the plate and incubate for 1 hour at $37^0C \pm 2^0C$.

7. Wash plate 5 times with the Working Wash Solution and an automated microwell plate washer. (Washer should fill wells to 400-450 µL volume and have a 30 sec. soak.) Tap plate dry after Wash.

8. Add 100 µL Avidin-HRP conjugate to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor). Cover plate and incubate for 15 minutes at $37^0C \pm 2^0C$.

9. Wash plate as in step 7.

10. Prepare Working Substrate Solution. For each MWP, mix 12 mL Substrate A with 3 mL Substrate B. Protect Working Substrate from direct light.

11. Pipette 100 µL of Working Substrate Solution into each well of the MWP (Program 2, AMPLICOR™ Electronic Impact Pipettor).

12. Allow color to develop for 10 minutes in the dark, at room temperature,

13. Add 100 µL of Stop Reagent to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor).

14. Measure the optical density at 450 nm (single wavelength) within 10 minutes.

E. Calculations:

1. Choose the appropriate HIV well OD values as follows:

   a. The HIV wells in rows A-F represent neat, 1:5, 1:25, 1:125, 1:625, and 1:3125 dilutions of the amplification products, respectively. The absorbance values should decrease with the serial dilutions, with the highest value for each test in row A and the lowest value in row Fa.

b. Choose the well with the lowest OD reading in the range of 0.200 to 2.00 OD units.

c. If any of the following conditions exist, see Unexpected Results Section.

all HIV OD values <0.200
all HIV OD values >2.000
HIV OD values not in sequence (i.e.,, OD values do not decrease from well A to well F)

2. Subtract background from the selected HIV OD values. Background = 0.070 OD units. Then calculate the Total HIV OD by multiplying the resulting OD value by the dilution factor associated with that well.

Total HIV OD = (Selected HIV OD-0.070)X dilution factor

3. Choose the appropriate QS well as follows:

a. The QS wells in rows G and H represent neat and 1:5 dilutions of the amplification products, respectively. The absorbance value in row G should be greater than the value in row H.

b. Choose the well with the lowest OD in the range of 0.300 to 2.00 OD units.

c. If the following conditions exist, see Unexpected Results Section.

both QS OD values <0.300
both QS OD values >2.000
QS OD values not in sequence (i.e.,, OD values do not decrease from well G to well H)

4. Subtract background from the selected QS OD values. Background = 0.070 units. Then calculate the Total QS OD by multiplying the resulting OD by the dilution factor associated with that well.

Total QS OD = (Selected QS OD - 0.070) X dilution factor

5. Calculate HIV-1 RNA copies/mL plasma according to the formula below.

$$\text{HIV RNA copies/mL plasma} = \frac{\text{Total HIV OD}}{\text{Total QS OD}} \times \text{Input QS Copies per PCR Reaction} \times 40$$

Where:
Input QS copies per PCR reaction is the number of QS molecules introduced into each reaction and is specific to each lot of QS RNA. See the Amplicor HIV Monitor^{TM} Test

Data Card for the Input QS Copies per PCR Reaction. Verify that the QS RNA lot number matches the lot number on the Data Card.

40 = Correction factor to convert copies/PCR to copies/mL of plasma..

6. Unexpected Results

a. All HIV OD values <0.200. If all the HIV wells have OD values less than 0.200, but the QS wells have the expected values, use 0.200 as the HIV OD and 1 as the dilution factor, calculate the result as above, and report result as "No HIV RNA detected; less than... " (the calculated value).

b. All HIV OD values >2.000. If all HIV wells have OD values greater than 2.000, then the HIV copy number is above the dynamic range of the assay. Repeat the entire test (including extraction), first diluting plasma 1:50 with HIV Negative Human Plasma. Calculate the HIV result as above, then multiply the final result by 50.

c. HIV OD values out of sequence. If the HIV wells do not follow the general pattern of decreasing OD values from well A to well F, then an error in dilution may have occurred. Repeat the assay on that sample.

1) In reactions containing a very high HIV copy number, the 1:1 and 1:5 wells can become saturated, turning a greenish-brown color before addition of Stop Solution and a brown color after addition of Stop Solution, resulting in lower ODs.

2) Very high OD values (>3) and very low OD values (<0.1) may not follow a pattern of decreasing OD values from well A to well F.

3) A significant deviation from the general rule of decreasing OD values from well A to well F indicates an error.

4) Both QS OD values <0.300. If both QS wells have OD values less than 0.300, then either the processed sample was inhibitory to the amplification or the RNA was not recovered from the sample. Repeat the assay for that sample including extraction.

5) Both QS OD values >2.000. If both QS wells have OD values greater than 2.000, then an error occurred. Repeat the assay for that sample including extraction.

6) QS OD values out of sequence. If the absorbance of well H is greater than the absorbance in well G, then an error occurred. Repeat the assay for that sample including extraction.

VI. QUALITY CONTROL

VQA standards must be run on the first plate of every assay. Control values are not valid if the QS and HIV absorbance values do not meet criteria described in the Calculation section, or the control results are n valid. The negative control should yield HIV OD values of less than 0.2 OD units in all HIV wells. The positive controls should yield final results that can be found on the Data Card, and should be checked for each lot number (when Roche Controls are used). When VQA standards are used, Controls should yield final results of 1.5×10^4, 1.5×10^5 and 7.5×10^5 copies/mL

A quality control log for the thermal cycler must be completed at the end of every run and show no errors.

VII. REPORTING RESULTS

Results are reported as RNA copies /mL, unless they are too low or too high. If all patient OD wells are <0.20, the result is reported as "No HIV-1 RNA detected, less than... (the calculated minimum value, see V, E, 6, a.) Test results greater than 750,000 copies/mL should be reported as "Greater than 750,000 copies /mL". If quantitation is desired, the original plasma must be diluted and repeated (see V. E, 6, b).

VIII. PROCEDURES NOTES

Racks which hold the processed plasma samples, and the MicroAmp® tray, retainer and base should be soaked overnight in 10% bleach after use to remove any DNA that may be present. Working surfaces in all areas should be cleaned with 10% bleach before and after use. To minimize contamination, the Amplicor HIV Monitor™ procedure must be carried out in the three separate areas, and a uni-directional traffic pattern (Reagent Preparation, Specimen Preparation, Amplification, Detection) must be observed. Use dedicated pipettors and consumables in each area.

IX. REFERENCES

Roche AMPLICOR HIV MONITOR™ TEST Procedure Manual

NASBA™ HIV-1 RNA QT
QUANTITATIVE RNA ASSAY

I. PRINCIPLE

Acquired Immunodeficiency Syndrome (AIDS) is an immunosuppressive disorder characterized by depletion of the CD4+ T cell population. A progressive, severe immunodeficient state is accompanied by a broad variety of clinical manifestations including opportunistic infections, an array of malignancies, and the frequent presence of neurological disorders.

The etiologic agent of AIDS is the Human Immunodeficiency Virus (HIV). It is transmitted by sexual contact, through contaminated injection needles, or through administration of contaminated blood or blood products. HIV is also capable of passing through the placenta. So far, two types of HIV have been found to cause AIDS: HIV-1, first isolated in 1983, and HIV-2, a second distinct but related type, first isolated in 1985.

The conventional method for detection of HIV infection is through serologic identification of an immunologic response to HIV, by means of enzyme-linked immunosorbent assays (ELISA), and confirmation of the results with more specific assays (western blot).

Unlike these indirect methods, nucleic acid amplification techniques such as Reverse Transcriptase PCR and NASBA™ do not depend on the development of an immunologic response to HIV, which occasionally takes six months or more from the time of infection to occur. They directly test for the presence of HIV RNA and thus can detect HIV infection before seroconversion. In addition, they are more sensitive than p24 antigen assays. The advantage of NASBA™ over Reverse Transcriptase PCR is that it requires no separate reverse transcriptase step or, as amplification is isothermal, any thermocycler equipment.

Nucleic acid amplification is also suitable for the quantitation of HIV-1 RNA in plasma and serum samples. Determination of the viral load appears to be a valuable marker for the prediction of disease progression and for monitoring the efficacy of anti-viral therapy especially in the early stage of the disease when conventional markers are often negative.

Quantitation with NASBA™ HIV-1 RNA QT is based on co-amplification of HIV-1 sample RNA together with internal calibrators, a technique which has proved to be superior to other quantitation methods. The quantity of amplified RNA is measured by means of electrochemiluminescence (ECL).

The NASBA™ HIV-1 RNA QT assay comprises four separate stages: Nucleic acid release, Nucleic acid isolation, Nucleic acid amplification, and Nucleic acid detection.

The nucleic acid release step is accomplished by adding the sample to lysis buffer containing guanidine thiocyanate and Triton X-100. Any viral particles, RNases, and DNases present in the sample are disintegrated and nucleic acid is released.

The next step is nucleic acid isolation. Three synthetic RNAs (Qa,Qb,Qc) of known high, medium, and low concentration, respectively, are added to the lysis buffer containing the released nucleic acid. These RNAs serve as internal calibrators, each differing from the HIV-1 wild type (WT) RNA by only a small sequence. Under high salt conditions, all nucleic acid in the buffer, including the calibrators, is bound to silicon dioxide particles. These particles, acting as the solid phase, are washed several times. Finally, the nucleic acid is eluted from the solid phase.

Nucleic acid amplification follows the nucleic acid isolation step. Any wild type HIV-1 RNA present in the eluted nucleic acid is co-amplified with three internal calibrators. Amplification is based on primer extension: the wild type RNA and the calibrator RNAs serve as templates for the extension of gag region primer 1 (containing the T7-RNA polymerase recognition site) by Avian Myeloblastosis Virus reverse transcriptase (AMV-RT). Extension is followed by degradation of the template RNAs by RNase H, synthesis of the second DNA strand through extension of primer 2 by AMV-RT, and RNA synthesis by T7-RNA polymerase. With RNA synthesis the system enters the isothermal cyclic phase, resulting in the accumulation of wild-type and calibrator RNA amplificates.

The final step is nucleic acid detection. Detection of HIV-1 RNA in a sample is based on the NASBA™ QR System electrochemiluminescence principle. To separate the amplificates (WT,Qa,Qb, and Qc), aliquots of the amplified sample are added to four hybridization solutions, each specific for one of the amplificates. Here, the respective amplificates are hybridized with a bead-oligo (i.e., a biotin -oligo bound to streptavidin coated magnetic beads acting as the solid phase) and a ruthenium-labeled probe. The magnetic beads carrying the hybridized amplificate/probe complex are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the electrochemiluminescence (ECL) reaction. The light emitted by the hybridized ruthenium labeled probes is proportional to the amount of amplificate. Calculation based on the relative amounts of the sample amplificates reveals the original amount of wild-type HIV-1 RNA in the sample.

II. SPECIMEN REQUIREMENTS

Plasma is the specimen of choice, although serum may be used. No special preparation or fasting of the patient is necessary.

1. Transfer greater than 2 mL of blood into an EDTA, citrate or heparin vacutainer.

2. Prepare plasma as soon as possible, i.e., preferably within two hours after collection, by centrifuging the capped tube for 15 $\pm$ 1 minutes at 1000 g.

3. Do not uncap tubes in the presence of other open tubes containing patient material.

4. Add specimens within two hours after collection to the lysis buffer tubes to avoid HIV-1 RNA degradation, or store immediately at -70^{O}C as a back-up. In lysis

buffer, the HIV-1 RNA can be stored at room temperature (15-30^{o}C) for a maximum of one day; for a maximum of one week at 2-8^{o}C; long-term storage is possible at -20^{o}C or, preferably, at -70^{o}C.

Note: The following should be considered unacceptable:

Whole unseparated blood.
Grossly lipemic or hemolyzed samples.
Specimens repeatedly frozen and thawed or those containing particulate matter may give erroneous results.

III REAGENTS

A. Precautions

Alterations in the physical appearance of the test kit material may indicate instability or deterioration. Expiration dates shown on component labels indicate the date beyond which components should not be used. Prepare reagents before starting nucleic acid release, isolation, amplification and detection, respectively.

AVOID CONTAMINATION: STORE AND PREPARE REAGENTS FOR NUCLEIC ACID RELEASE, ISOLATION, AMPLIFICATION AND DETECTION AT THE LABORATORY AREAS WHERE NUCLEIC ACID RELEASE, ISOLATION, AMPLIFICATION AND DETECTION, RESPECTIVELY, ARE TO BE PERFORMED.

Make sure reagents and samples are at room temperature (15-30^{o}C) before starting nucleic acid release, isolation, amplification and detection, respectively.

Before opening a tube which contains lyophilized material , make certain that it is at the bottom of the tube.

Use prepared reagents immediately. Storage of prepared or opened reagents is not recommended.

CAUTION: The lysis buffer and the wash buffer contain guanidine thiocyanate. Guanidine thiocyanate is harmful by inhalation, in contact with skin and if swallowed. Contact with acids liberates very toxic gas. The elution buffer, the enzyme diluent and the detection diluent contain sodium azide. When discarding into sewerage, always flush with copious quantities of water. This helps prevent formation of metallic azides which, when highly concentrated in metal plumbing, may be potentially explosive. Plumbing should be periodically decontaminated according to appropriate guidelines.

B. The NASBA™ HIV-1 RNA QT provides the following reagents.

1. The regents needed to perform 50 NASBA™ HIV-1 RNA QT tests are supplied in three separate boxes containing: lysis buffer, reagents for nucleic acid isolation and amplification, and reagents for nucleic acid detection.

   a. Nucleic Acid Release Reagent

      1) Lysis Buffer: 50 tubes (0.9 mL/tube) 5 mol/L guanidine thiocyanate, Triton X-100, Tris/HCl. Store at 2 -8^{0}C. Note manufacturer's outdate.

   b. Nucleic Acid Isolation

      1) Wash Buffer: 5 vials (22 mL/vial) 5 mol/L guanidine thiocyanate, Tris/HCl. Store at 2 -8^{0}C. Note manufacturer's outdate.

      2) Silica: 5 tubes (0.8 mL/tube). Store at 2 - 8^{0}C. Note manufacturer's outdate.

      3) Elution Buffer: 5 tubes (1.5 mL/tube) Tris/HCl Preservative: 0.9 g/L sodium azide. Color Code: White. Store at 2 -8^{0}C. Note manufacturer's outdate.

      4) Calibrator: 5 foiled-packed tubes (6.5 mg/tube) lyophilized synthetic RNA (Qa, QB, and Qc) sphere; each tube contained in a foil pack with silica gel desiccant. Color Code: Yellow. Store at 2 -8^{0}C. Note manufacturer's outdate.

   c. Nucleic Acid Amplification Reagents

      1) Enzymes: 5 foil-packed tubes (20 mg/tube) lyophilized AMV-RT (chicken), RNase H T7-RNA polymerase, BSA and nucleotides; each contained in a foil pack with silica gel desiccant. Color Code: Red. Store at 2 -8^{0}C. Note manufacturer's outdate.

      2) Enzyme Diluent: 5 tubes (0.2 mL/tube) Tris/HCl Preservative: 0.9 g/L sodium azide. Color Code: Red. Store at 2 -8^{0}C. Note manufacturer's outdate.

      3) Primers: 5 foil-packed tubes (0.5 mg/tube) lyophilized sphere with synthetic primers, dithiothreitol, KCl and $MgCl_2$; each tube contained in a foil pack with silica gel. Color Code: Blue. Store at 2 -8^{0}C. Note manufacturer's outdate.

      4) Primer Diluent: 5 tubes (0.2 mL/tube) Tris/HCl, 30% DMSO Color Code: Blue. Store at 2 - 8^{0}C. Note manufacturer's outdate.

d. Nucleic Acid Detection

1) Bead-oligo: 2 tubes (1.68 mL/tube) biotin-oligo bound to streptavidin-coated magnetic beads. Preservative: 1 g/L 2-chloro-acetamide. Color Code: Lilac. Store at 2 -8^{0}C. Note manufacturer's outdate.

2) WT Probe: 1 tube (0.84 mL) Ruthenium-labeled oligo. Preservative: 5g/L 2-chloro-acetamide. Color Code: White. Store at 2 -8^{0}C. Note manufacturer's outdate.

3) Qa Probe: 1 tube (0.84 mL) Ruthenium-labeled oligo. Preservative: 5 g/L 2-chloro-acetamide. Color Code: Red. Store at 2 -8^{0}C. Note manufacturer's outdate.

4) Qb Probe: 1 tube (0.84 mL) Ruthenium-labeled oligo. Preservative: 5 g/L 2-chloro-acetamide. Color Code: Yellow. Store at 2 -8^{0}C. Note manufacturer's outdate.

5) Qc Probe: 1 tube (0.84 mL) Ruthenium-labeled oligo. Preservative: 5g/L 2-chloro-acetamide. Color Code: Blue. Store at 2 -8^{0}C. Note manufacturer's outdate.

6) Detection Diluent: 1 vial (8.4 mL) Tris/HCl. Preservative: 0.9 g/L sodium azide. Store at 2 - 8^{0}C. Note manufacturer's outdate.

7) Instrument Reference Solution: Streptavidin- coated magnetic beads. Store at 2 - 8^{0}C. Note manufacturer's outdate.

B. Additional reagents required but not provided are:

1. 70% Ethanol (prepared from 95% ethanol, ACS quality); prepare tubes of 12 mL each and store at room temperature (15-30^{O}C).

2. Acetone (analytical grade); prepare tubes of 12 mL each and store at room temperature (15-30^{O}C).

IV. EQUIPMENT AND SUPPLIES

A. Nucleic Acid Release

EDTA, citrate, or heparin vacutainer tubes.
Centrifuge suitable for vacutainer tubes.
Tubes for plasma or serum storage (1-5 mL).
Disposable transfer pipettes (5 mL).

Absorbent tissue.

B. Nucleic Acid Isolation

RNase free 1.5 mL test tubes (preferably screw-cap), which can be centrifuged at 10,000 g, and 1.5 mL test tube racks.
Calibrated disposable pipette tips (10, 100, (2X) 1000 µL) with aerosol resistant tips.
Heating block (or water bath) capable of heating 1.5 mL test tubes to 56 $\pm$ 1°C.
Timer.
Vortex.
Table top centrifuge for 1.5 mL test tubes (capable of 10,000 g.)

C. Nucleic Acid Amplification

Calibrated disposable tip pipettes (10 and 100 µL) with aerosol resistant tips.
Two heating blocks (or water baths) capable of heating 1.5 mL test tubes to 41 $\pm$ 0.5°C and 65 $\pm$ 1° C.
Two calibrated mercury thermometers (precision $\pm$ 0.2°C).
Water bath capable of heating 1.5 mL test tubes to 41 $\pm$ 0.5°C
Timer.
Vortex.
Table top centrifuge for 1.5 mL test tubes (capable of 10,000 g).
1.5 mL test tube racks.

D. Nucleic Acid Detection

Calibrated disposable tip pipettes for variable volumes (5 to 1000 µL) with corresponding tips.
Heating block (or water bath) capable of heating 1.5 mL test tubes to 41 $\pm$ 0.5°C.
Timer.
Vortex.
Table top centrifuge for 1.5 mL test tubes (capable of 10,000 g).
RNase free 1.5 mL test tubes (preferably screw-cap), which can be centrifuged at 10,000 g, and 1.5 mL test tube racks.
NASBA™ QR System with PC, Assay Buffer and Cleaning Solution.
Polypropylene tubes (5 mL) for hybridization (250 tubes per kit).
Repeating pipette with disposable tips (0.500 mL and 5.00 mL).
Vials (50 mL) with screw cap for disposal of pipette tips.
Shaker capable of 1100 rpm.
Adhesive tape.

V. PROCEDURE

A. Nucleic Acid Release

1. Preparation of Lysis Buffer

a. Prewarm lysis buffer tubes for about 30 minutes (suggested temperature: 37^{O}C) before starting the assay to make sure that any crystals in the lysis buffer have dissolved.

b. Protect the lysis buffer from excessive light.

2. Nucleic Acid Release Procedure

a. Spin lysis buffer tubes (i.e., centrifuge the lysis buffer tubes at a speed and time required to spin all fluid down to the bottom of the tubes e.g., 15 seconds at 10,000 g).

b. Uncap tube containing prepared specimen (refer to section II.).

c. Transfer 100 µL of the prepared specimen into a lysis buffer tube. NOTE: smaller specimen volumes from 10-100 µL may also be used. Note that use of smaller specimen volumes has effect on the interpretation of results.

d. Store residual specimen in a separate storage tube (for possible use in other serological tests) at -20^{O}C or preferably -70^{O}C.

e. Close tubes.

f. Vortex lysis buffer.

g. Prepare next sample in the same way.

Note: The lysis buffer tubes with the sample can be stored at room temperature for 24 hrs, 7 days at 2-8^{0}C, and long term storage at -70^{0}C.

B. Nucleic Acid Isolation

1. Preparation of Nucleic Acid Isolation Reagents

a. Prewarm wash buffer tubes for about 30 minutes (suggested temperature: 37^{O}C) before starting the assay to make sure that any crystals in the wash buffer have dissolved.

b. Protect the wash buffer from excessive light.

c. Vortex the silica tube before starting the isolation procedure until an opaque suspension is formed. The silica particles will settle again loosely. Vortex again before each pipetting step.

d. Reconstitute the calibrator (yellow) in 220 µL elution buffer (white).

e. Vortex the solution.

Note: Use within one hour after preparation. Number of calibrator molecules per 20 µL: Qa 10^6, Qb 10^5, Qc 10^4.

2. Nucleic Acid Isolation Procedure

a. Spin lysis buffer tubes.

b. To each lysis buffer tube add 20 µL calibrator solution, vortex, and spin the lysis buffer tubes.

c. To each tube add 50 µL of vortexed silica suspension.

d. Leave the tubes for 10 $\pm$ 1 minutes at room temperature (15-30^{o}C). NOTE: VORTEX LYSIS BUFFER TUBES REGULARLY, E.G., EVERY TWO MINUTES, TO PREVENT SILICA FROM SETTLING ON THE BOTTOM.

e. Spin lysis buffer tubes.

f. Remove the supernatant from the lysis buffer tubes with a 1000 µL disposable tip pipette, using a fresh sterile tip for each tube; avoid whirling up the pellet.

g. Wash the silica pellet in the lysis buffer tubes five times i.e., :

1) twice (2X) with wash buffer
2) twice (2X) with 70% ethanol
3) once (1X) with acetone

h. Wash Procedure:

1) Add 1 mL of wash buffer/ethanol/acetone to each lysis buffer tube with a **second** 1000 µL disposable tip pipette, using a fresh sterile tip for each lysis buffer tube.

2) Vortex until pellets are resuspended.

3) Spin lysis buffer tubes.

4) Remove the supernatant with a 1000 µL tip pipette, using a fresh sterile tip for each lysis buffer tube.

5) Use a clean tube rack for next wash.

i. Dry the silica pellets in open lysis buffer tubes at 56^{o}C for 10 $\pm$ 1 minutes in a heating block.

NOTE: COVER THE LYSIS BUFFER TUBES WITH TISSUE TO AVOID CONTAMINATION.

j. Check if lysis buffer tubes are dry (the silica pellet will be white). If tubes are dry add 50 µL elution buffer to each lysis buffer tube and resuspend the silica pellets by vortexing.

k. Leave the resuspended silica for 10 $\pm$ 1 minutes at 56^{o}C to elute the nucleic acid. NOTE: VORTEX EVERY 2 MINUTES DURING ELUTION.

l. Centrifuge lysis buffer tubes 2 minutes at 10,000g.

m. Transfer 5 µL of the supernatant nucleic acid from the lysis buffer tube to a fresh test tube.

n. Store remaining 45 µL supernatant with silica pellet as a back-up at -20^{o}C or, preferably, at -70^{o}C.

o. Transfer the test tubes containing the 5 µL supernatant nucleic acid to the amplification lab area.

p. If the test tubes are not to be immediately used for amplification, store at -20^{o}C or, preferably, at -70^{o}C.

C. Amplification

1. Preparation of Amplification Reagents

a. Add 45 µL enzyme diluent (red) to the lyophilized enzymes (red).

b. Leave for 15 minutes; ensure complete reconstitution by gently "rolling" the closed tube every 5 minutes between your fingers.

NOTE: USE WITHIN 1 HOUR AFTER PREPARATION. DO NOT VORTEX.

c. Add 120 µL primer diluent (blue) to the lyophilized primers (blue) and vortex. NOTE: USE WITHIN 1 HOUR AFTER PREPARATION.

2. Amplification Procedure

NOTE: AMPLIFICATION STEP a. TO d. MUST BE PERFORMED IN A FUME HOOD TO REDUCE THE RISK OF CONTAMINATION; STEP f. and g. SHOULD PREFERABLY ALSO BE PERFORMED IN A FUME HOOD.

a. Add 10 µL of primer solution to each test tube containing 5 µL supernatant nucleic acid and then close the tube.

b. Incubate test tubes for 5 $\pm$ 1 minutes at 65 $\pm$ 1^{o}C.

c. Cool test tubes for a minimum of 5 minutes at 41 $\pm$ 0.5^{o}C.

d. Mix enzyme solution by "rolling" the closed tube between your fingers. Add 5 µL of enzyme to each test tube. Mix gently by tapping the test tube and incubate tubes for at least 5 minutes at 41 $\pm$ 0.5^{o}C.

NOTE: AVOID ANY UNNECESSARY DELAY BETWEEN THIS INCUBATION AND INCUBATION IN STEP f. BELOW TO PREVENT DISCONTINUATION OF THE AMPLIFICATION PROCESS.

e. Transfer tubes to the detection area and spin the test tubes.

f. Incubate the test tubes at 41 $\pm$ 0.5^{o}C for 90 $\pm$ 5 minutes, using a water bath.

g. Store test tubes that are not to be immediately used for detection up to 1 month at -20^{o}C.

D. Detection

1. Preparation of Detection Reagents

a. For Hybridization solution 1, vortex bead-oligo (lilac) until an opaque solution is formed. Immediately after vortexing, add 130 µL bead-oligo to a fresh tube and add 130 µL WT probe (white).

b. For hybridization solutions 2 to 4 follow the same procedure, replacing the WT probe by Qa (red), Qb (yellow) and Qc (blue) probe, respectively.

c. Vortex hybridization solutions before use.
d. Add 1.3 mL detection diluent to a fresh test tube.

2. Detection Procedure

NOTE: DETECTION SHOULD PREFERABLY BE PERFORMED IN A FUME HOOD TO REDUCE THE RISK OF CONTAMINATION.

a. For each amplified sample, place four fresh 5 mL polypropylene tubes ready for use in a rack (referred to as hybridization tubes 1 to 4 in the following). Place one additional fresh 5 mL polypropylene tube ready for use as a blank (i.e., detection diluent instead of amplified sample is added to this tube).

b. Vortex hybridization solutions until an opaque solution is formed.

c. Add 20 µL of hybridization solution 1 to each hybridization tube 1 and to the first blank, using a repeating pipette.

d. Add 20 µL of hybridization solution 2 to each hybridization tube 2 using a repeating pipette.

e. Add 20 µL of hybridization solution 3 to each hybridization tube 3 using a repeating pipette.

f. Add 20 µL of hybridization solution 4 to each hybridization tube 4 using a repeating pipette.

g. For each amplified sample, add 100 µL detection diluent to a fresh test tube using a repeating pipette.

h. Close the tubes containing detection diluent.

i. For each amplified sample:

1) Open one of the test tubes containing detection diluent.
2) Add 5.0 µL amplified sample.
3) Close test tube and vortex.
4) Spin test tube.

j. For each amplified sample (diluted in step I.):

1) Add 5.0 µL diluted amplified sample to each of the four hybridization tubes, using a fresh pipette tip for each addition.

NOTE: AVOID CONTACT OF THE PIPETTE TIP WITH THE INSIDE WALL OF THE HYBRIDIZATION TUBES. THE TIP SHOULD ONLY TOUCH THE HYBRIDIZATION SOLUTION IN THE TUBES. USE 50 mL VIALS WITH SCREW CAPS FOR DISPOSAL OF THE PIPETTE TIPS TO REDUCE CONTAMINATION RISKS.

2) Add 5.0 µL detection diluent to each of four blanks.
3) Mix hybridization tubes and blanks until an opaque solution is formed (either mix simultaneously, e.g., using a shaker (1100 rpm), or vortex one by one).
4) Cover hybridization tubes and blanks with adhesive tape.

k. Incubate all hybridization tubes and blanks for 30 $\pm$ 1 minutes at 41 $\pm$ 0.5^{o}C for hybridization.

NOTE: DURING HYBRIDIZATION, MIX HYBRIDIZATION TUBES AND BLANKS EVERY 10 MINUTES.

l. Add 300 µL NASBA™ QR System Assay Buffer (refer to NASBA™ QR System Operator's Manual) to each hybridization tube and blank, using a repeating pipette. In addition, add 300 µL of instrument reference solution to a fresh 5 mL polypropylene tube.

NOTE: THE TUBES CAN NOW BE IDENTIFIED BY THE COLOR OF THEIR CONTENT (WT= COLORLESS; Qa = LIGHT BLUE; Qb = MEDIUM BLUE; Qc = DARK BLUE).

m. Perform any required NASBA™ QR System maintenance procedures (refer to Section VI.).

n. Place all hybridization tubes and blanks on the instrument carousel (for positioning, refer to Section VI.). Instrument reference solution tube should be placed in position #1 of the carousel. The Hybridization blank should be placed in position #2 of the instrument carousel.

o. Run assay (refer to Section VI. for creating, editing, and running of the job list).

VI. INSTRUMENTATION

A. Entering System

1. Log on to the NASBA™ QR System by selecting the appropriate user name from those displayed on the screen. Enter your password and the system will allow you access to the main menu.

B. Creating Work list

1. Select "ROUTINE" from the main menu and then select the subtopic "New Run." A different screen will then be displayed.

2. Select "HIV Quantitation" from this new screen. Select which type of calibrator that was used in the assay (diluted or concentrated).

3. Enter the individual samples and after each select the “Add to List" option. Keep adding samples until all the samples are entered or you reach the end of the Work list (maximum of 12 samples/Work list).

C. Loading Machine

1. Remove the carousel from the reader by releasing the cord from the top of the carousel and then release the carousel from the reader by pushing the unlocking lever to the right.

2. Remove the cover on top of the carousel by sliding it to the side. The carousel is now ready for loading.

3. Load the carousel as follows:

POSITION	CONTENTS
1	REFERENCE SOLUTION
2	ASSAY NEGATIVE CONTROL
3	WT TUBE PATIENT ONE
4	Qa TUBE PATIENT ONE
5	Qb TUBE PATIENT ONE
6	Qc TUBE PATIENT ONE
7	WT TUBE PATIENT TWO
8	Qa TUBE PATIENT TWO
9	QB TUBE PATIENT TWO
10	Qc TUBE PATIENT TWO

4. Continue this pattern until all patient tubes have been put in the carousel.

5. After all tubes have been loaded into the carousel, place the carousel on the reader and push until a click is heard. Reconnect the cord to the top of the carousel.

D. Running Work list

1. Select "Run Work list". The carousel should spin to read position one in about 30 seconds, if it doesn't consult operating manual for explanation.

2. After last tube has been read and the machine has become idle, the results of the assay can be displayed and printed. Select "Assay results" from the menu and the results will be displayed on the screen. Select the Print option at the bottom of the screen for a hard copy of the Work list's data.

E. Saving the Run

1. Select "Save Run as" and the system will prompt you to accept the name the system has selected or give you the option to name the filename yourself.

VII. RESULTS

The NASBA™ QR System automatically calculates the original HIV-1 RNA plasma or serum level in the volume of sample added to the lysis buffer (for viewing, printing, storing, or retrieving of results refer to Section VI.).

There are three types of results obtained from the reader: Valid, Warning, and Invalid.

A. Valid Result-A valid result can be reported normally without any further analysis.

B. Warning Result-A Warning result is one that can be reported after further analysis of the assay result or is one that may have to be repeated. A sample that gets a "warning" result can usually fall into one of two categories. The first is the Qa standard is above the threshold of the reader, this will be displayed as the Qa value of 9999999. This result is caused by exceptional amplification of both standards and patient RNA. If the patient value (WT signal) falls between the two other standards values then the result can be reported out. If the patient value (WT signal) is outside this range then the RNA detection section of the procedure must be repeated with a slight alteration in the procedure. This slight change is: increase the original dilution of the sample that was subjected to the detection process. (i.e., if the original dilution was 5 µL patient sample into 100 µL diluent (1:21), then increase dilution to 1:41 by adding 2.5 µL patient sample into 100 µL diluent).The second reason is that the standard curve that the sample is being evaluated on isn't "perfect". This problem doesn't need to be corrected if there is no WT signal (i.e., if the WT signal is 100 or less, which corresponds to an answer of "Less than Lower Limit); that answer could go out normally. If the WT signal is greater than 100, the problem

usually can be resulted by decreasing the hybridization dilution (i.e., if original dilution was 1:61 then decrease dilution to 1:10 and rehybridize and redetect).

C. An Invalid result cannot be reported under any circumstance. This result usually causes the sample to be repeated on the next run. If the result was invalid because the ratio(s) between one or several of the standards is incorrect (too low or too high), then repeat the detection part of the procedure. If upon completion of the repeat detection the sample is still invalid, the sample must undergo the complete procedure on the next run. If the signals were all "1"s then the entire procedure must be completed so a valid answer can be obtained for the patient.

D. If a sample is repeated and still produces an "Invalid" result, then the physician will be notified and a free text message will be entered with the worksheet.

VIII. INTERPRETATION OF RESULTS

Present data are insufficient to establish a direct correlation between HIV-1 RNA levels and the clinical outcome of the disease. However, studies reveal that HIV-1 RNA levels are associated with disease stage and CD4+ T cell counts. In addition, levels change rapidly in response to effective therapy.

With primary infection, HIV-1 RNA levels reach a peak (coinciding with seroconversion) within days or weeks after onset of symptoms and decline rapidly thereafter. Primary infection is followed by an asymptomatic stage and later by persistent lymphadenopathy, where levels remain relatively low. With progression to AIDS-related complex, levels increase significantly. Another significant rise in levels comparable to those in primary infection is observed after progression to AIDS.

Throughout disease progression, a rise in HIV-1 RNA levels is significantly correlated to a decline in CD4+ T cell count.

High individual peak levels in primary infection and persisting high levels during the following stages may imply a negative prognosis.

In a subgroup of patients, treatment with zidovudine (AZT) is followed by a rapid decrease in HIV-1 RNA levels. A rapid rebound to pretreatment levels is observed after discontinuation of therapy, suggesting continuing viral replication throughout treatment. Other patients respond only transiently to therapy or do not respond at all. Quantitation of HIV-1 RNA levels can help to identify non-responders and spare them the consequences of ineffective AZT therapy.

IX. PROCEDURAL NOTES

Perform nucleic acid release, isolation, amplification and detection in separate laboratory areas.

Air from the detection area must not be allowed to enter the other areas (detection should be performed in a fume hood).

Keep all tubes and vials closed when not in use.

Do not use pipettes and other equipment which have been used in one laboratory area in the other areas.

Use a fresh pipette or pipette tip for each pipetting action.

Use pipettes with aerosol resistant tips or air displacement pipettes for fluids possibly containing nucleic acid.

Pipetting solutions always must be performed out of or into an isolated tube that is opened and closed exclusively for this action. All other tubes and vials should be kept closed and separated from the one handled.

Use disposable gloves when working with clinical material possibly containing HIV-1 or amplified material. If possible, change gloves after each pipetting step in the test procedure, especially after contact with possibly contaminated material.

In the amplification lab area, the use of water baths instead of heating blocks increases the risk of contamination.

Soak tube racks used during nucleic acid isolation in a detergent (EXTRAN-1000) for at least one hour after each test run.

All reagents and specimens must be mixed thoroughly before use. NOTE: ENZYMES MUST NOT BE VORTEXED. MIX GENTLY, E.G., BY TAPPING THE TUBE.

X. LIMITATIONS OF THE PROCEDURE

A negative test result does not exclude the possibility of exposure to, or infection with, HIV-1.

The assay must be performed in strict accordance with the instructions in this package insert and the NASBA™ QR System Operator's Manual to obtain accurate, reproducible results.

Samples from individuals infected with HIV-2 may exhibit cross-reactivity.

In addition to quantitative HIV-1 RNA load, other virologic or immunologic factors may contribute to variable rates of CD4+ T cell counts and clinical outcome of the disease.

XI. REFERENCES

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CODON 215 MUTATION PCR DETECTION ASSAY FOR HIV DNA

I. PRINCIPLE

The Codon 215 Test is a polymerase chain reaction (PCR)-based test designed to detect the presence of a specific DNA sequence at Codon 215 in the reverse transcriptase (RT) gene of human immunodeficiency virus (HIV). HIV isolates possessing a DNA sequence of TAC or TTC at codon 215 show reduced susceptibility to zidovudine. Clinical studies have indicated that patients with HIV-1 possessing these "mutant" sequences have a greater risk for clinical progression than patients with the "wildtype" sequence (ACC).

In this test, clinical samples are processed and a portion of the HIV RT gene is amplified by PCR. A second, selective round of two PCR reactions is then conducted in parallel using PCR product from the first reaction: one reaction will amplify wildtype codon 215 sequences, and one reaction will amplify mutant sequences (if any) at that position. Both second round reactions are then run on an agarose gel to determine which second round successfully amplified, indicating the genotype of the virus tested reaction (wildtype or mutant).

II. SPECIMEN REQUIREMENTS

HIV-positive cultured cells or uncultured PBMC are suitable specimens.

A. Cultured PBMC. Add approximately 1 to 2 x 10^6 cells to a siliconized 1.5 mL microcentrifuge tube. Centrifuge the tube for approximately 1 min at 10,000 rpm. Remove and discard the supernatant and store the cell pellet at -20^0C or lower.

B. Uncultured PBMC. PBMC isolated from whole blood either by Ficoll-Hypaque density gradients or treated by direct lysis using ammonium chloride (Roche Sample Preparation Reagent, or other similar lysing procedure are acceptable) may be used. Pellets are suitable for use or storage when no erythrocytes are macroscopically visible. Pellets from approximately 0.5 mL of whole blood or pellets of 1 to 2 x 10^6 Ficoll-Hypaque isolated PBMC should be stored at -20^0C or lower. PBMC isolated from heparinized blood are unreliable for PCR tests, as are pellets containing significant numbers of erythrocytes.

III. REAGENTS

Cell Lysis Buffer: A 10-50 mM Tris-HCl solution (pH 8.3) containing 2.5 mM magnesium chloride, 25 mM potassium chloride, and 100 µg/mL proteinase K. Aliquots should be stored at -20^0C or lower.

PCR Reaction Buffer: A 10-50 mM Tris-HCl solution (pH 8.3) containing 25 mM potassium chloride, 1.5 to 2.25 mM magnesium chloride, and 0.1 mg/mL gelatin. Aliquots should be stored at -20^0C or lower.

Taq DNA polymerase (2.5 units/mL), stored at -20^{0}C or lower.

Deoxynucleotide (dNTPs): A solution containing 25 mM each of deoxyguanosine (dGTP), deoxyadenosine (dATP), deoxycytosine (dCTP), deoxythymidine (dTTP). Aliquots should be stored at -20^{0}C or lower.

PCR primer "A'": A solution containing the oligomer
5'-TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "NE1'": A solution containing the oligomer
5'-CTTACTAACTTCTGTATGTCATTGACAGTCCAGCT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "B": A solution containing the oligomer
5'-GGATGGAAAGGATCACC-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "215WT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGGT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "215MUT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGAA-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

Water: Distilled grade or higher, stored at room temperature.

Wildtype Control DNA: A solution containing DNA from the HIV RT gene containing the wildtype (ACC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

Mutant Control DNA: A solution containing DNA from the HIV RT gene containing the mutant (TAC or TTC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

TBE Buffer: A solution containing 0.089M Tris borate (pH 8.3) and 0.01M EDTA, stored at room temperature.

Agarose: Molecular-biology grade, stored at room temperature.

Molecular Weight Marker: 100 base-pair marker (or equivalent) for electrophoresis, stored at 4^{0}C.

Gel Loading Buffer: A solution of 30% glycerol containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, or equivalent, stored at 4^{0}C.

IV. SUPPLIES AND EQUIPMENT

PCR Reaction Tubes and Caps
Perkin-Elmer GeneAmp PCR 9600 Thermal Cycler (or equivalent)
Pipettors (20 µL, 200 µL, and 1000 µL)
Aerosol-resistant Pipette Tips
Vortex Mixer
Water Bath, Incubator, or Heat Block for 56^0C ($\pm 2^0$C) and 99^0C ($\pm 2^0$C)
Agarose Electrophoresis Apparatus
Camera
Ultraviolet light source
High Speed Film
Latex Gloves
Lab Coat

V. PROCEDURE

A. Sample Preparation.

1. Add 180 µL of water to the cell pellet and vortex.

2. Add 20 µL of Cell Lysis Buffer and incubate 1 to 24 hours at 56^0C.

3. Heat the lysate to 95^0C to 100^0C for 10 min to inactivate the proteinase K.

B. First Round PCR.

1. For each sample to be tested, prepare the following reaction mix:

   10 µL PCR Reaction Buffer
   1 µL dNTPs
   1 µL primer NE1'
   1 µL primer A'
   0.5 µL Taq polymerase
   66.5 µL water

2. Mix the reaction mix thoroughly and pipette 80 µL of the reaction mix to a PCR reaction tube.

3. Pipette 20 µL of the test sample into the reaction tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
35 cycles of:	94^0C for 30 sec
	55^0C for 30 sec
	72^0C for 60 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples can be stored at 4^0C for 24 hr or -20^0C or lower for longer periods.

C. Second Round PCR

1. For each sample to be tested, prepare the following reaction mixtures:

Wildtype:	10 µL PCR Reaction Buffer
	1µL dNTPs
	1 µL primer B
	2 µL primer 215WT
	0.5 µL Taq polymerase
	81.5 µL water

Mutant:	10 µL PCR Reaction Buffer
	1 µL dNTPs
	1 µL primer B
	2 µL primer 215MUT
	0.5 µL Taq polymerase
	81.5 µL water

2. Mix the reaction mixes thoroughly and pipette 95 µL of each reaction mix into separate PCR reaction tubes.

3. Pipette 5 µL of the first round PCR product into a wildtype reaction mix tube and another 5 µL into a mutant reaction mix tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
30 cycles of:	94^0C for 60 sec
	48^0C for 30 sec
	72^0C for 30 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples may be analyzed immediately or stored at 4^0C or lower.

D. Gel Electrophoresis

1. Prepare a 3% agarose gel containing 0.5 µg/mL ethidium bromide in TBE.

2. Pipette 5 µL of Gel Loading Buffer into microcentrifuge tubes.

3. Pipette 20 µL of each second-round PCR product into a tube and mix.

4. Load the samples carefully into adjacent wells on the gel. (Load 12 L in using the minigel setup). Include one lane of the appropriate amount of 100 bp ladder on each gel.

5. Run the gel in TBE for approximately 1 hr at 120V.

6. Remove the gel from the electrophoresis apparatus and photograph the gel under ultraviolet light.

VI. CALCULATIONS/DATA ANALYSIS

Samples which have a 210 base-pair product (band) in the wildtype reaction and do not have a 210 base-pair product in the mutant reaction are considered to have wildtype sequences at Codon 215. Samples which have a 210 base-pair product in the mutant reaction and do not have a 210 base-pair product in the wildtype reaction are considered to have mutant sequences at Codon 215. If a 210 base-pair product is detected in both reactions, the first round PCR product is diluted 1:1000, and 10 µL of the dilution is amplified in another second round PCR reaction. If a 210 base-pair product persists in the wildtype reaction but disappears from the mutant reaction, the sample is considered to have the wildtype sequence. If a 210 base-pair product persists in the mutant reaction but disappears from the wildtype reaction, the sample is considered to have the mutant sequence. If a 210 base-pair product persists in both wildtype and mutant reactions, the sample is considered to have a mixture of wildtype and mutant sequences at Codon 215.

VII. QUALITY CONTROL

For all amplifications, a reaction mix control consisting of reaction mix with water instead of test sample is required. The sample results for a given assay are invalid when a 210 base-pair band is detected in this control.

The second round PCR requires control reactions consisting of reaction mix with first-round PCR products of known wildtype and mutant viruses containing the 215 mutation. These reactions will validate the ability of the wildtype and mutant reactions to accurately detect wildtype or mutant DNA in the test samples. The sample results for a given assay are invalid when either of these controls do not yield the appropriate 210 base-pair product.

VIII. PROCEDURE NOTES

A. Work areas in which the different PCR steps are performed should be geographically separated. At least, the room in which the second-round PCR product is analyzed should be separate from the areas in which reaction mixes and samples are prepared. A better arrangement is to have a separate room for product analysis, sample preparation, and reaction mix preparation.

B. Good laboratory technique is essential to the proper performance of this assay. Extreme care should be taken to avoid the contamination of reagents with samples, controls, or PCR product. Any reagent suspected of contamination should be discarded.

C. All pipettors, tips, reagents, and lab coats should be dedicated to and kept in only one PCR area (e.g., pipettors for loading gels cannot be used to prepare samples or reagents; lab coats worn during loading gels should not be worn into the reagent preparation area).

D. Pipette tips with aerosol barriers should be used during all steps.

E. PCR reaction trays (if used) should be soaked in bleach, rinsed with water, and dried before being used for another set of reactions.

F. Operators should pay close attention when transferring first-round PCR product into second-round reactions. Transferring an inadequate volume to one of the two reactions (wildtype or mutant), completely missing one of the two reactions, or confusing the identity of sample tubes can provide incorrect results without the mistake being obvious.

IX. LIMITATIONS

A. As with any PCR-based test, accurate detection of the Codon 215 mutation depends on extreme care being taken by laboratory technicians in handling reagents and performing the test, to ensure that any results obtained are derived from the patient's specimen and not from external sources.

B. Samples collected in heparin-containing tubes, vials, or equipment interfere with PCR reactions, which often results in no amplification. To ensure the greatest possibility of obtaining results, specimens should be collected in non-heparin-containing vessels.

C. The presence or absence of Codon 215 mutations cannot independently predict or determine the course of HIV infection. The results of this test should only be interpreted in conjunction with other laboratory and clinical data available to the clinician.

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CODON 215 MUTATION PCR DETECTION ASSAY FOR HIV RNA

I. PRINCIPLE

The Codon 215 Test is a polymerase chain reaction (PCR)-based test designed to detect the presence of a specific DNA sequence at Codon 215 in the reverse transcriptase (RT) gene of human immunodeficiency virus (HIV). HIV isolates possessing a DNA sequence 215 of TAC or TTC at codon 215 show reduced susceptibility to zidovudine. Clinical studies have indicated that patients with HIV-1 possessing these "mutant" sequences have a greater risk for clinical progression than patients with the "wildtype" sequence (ACC).

In this test, clinical samples are processed and a portion of the HIV RT gene is amplified by PCR. A second, selective round of two PCR reactions is then conducted in parallel using PCR product from the first reaction: one reaction will amplify wildtype codon 215 sequences, and one reaction will amplify mutant sequences (if any) at that position. Both second round reactions are then run on an agarose gel to determine which second round successfully amplified, indicating the genotype of the virus tested reaction (wildtype or mutant).

II. SPECIMEN REQUIREMENTS

Serum, plasma, and seminal fluid are suitable specimens. Aliquots of 0.5 to 1.0 mL should be stored at -70^{0}C or lower. Plasma isolated from heparinized blood is unreliable for PCR tests, and should be avoided. Suitable anticoagulants are citrate, acid citrate dextrose, and EDTA.

III. REAGENTS

A. Reagents Unique for RNA Preparation Method A:

Solution D: A solution containing 5M guanidine thiocyanate, 0.033M sodium citrate (pH 7.0), 10% sarcosyl, and 0.007M 2-mercaptoethanol, stored at room temperature.

Sodium Acetate: A 3M solution (pH 4.5), stored at room temperature.

Yeast Transfer RNA, 100 µg/mL. Aliquots should be stored at -20^{0}C.

Phenol: Tris-saturated phenol (pH 8.0), stored at 4^{0}C.

Chloroform:Isoamyl alcohol: A 49:1 mixture of chloroform and isoamyl alcohol, stored at room temperature.

B. Reagents Unique for RNA Preparation Method B:

Tri-Reagent (Molecular Research Centers, Inc., Cincinnati, OH), stored at 4^0C.

Chloroform, stored at room temperature.

C. Common Reagents

Viral Lysis Buffer: A solution containing 2 mm dithiothreitol, 0.1% Noniodet P-40, 0.01% RNAsin (or equivalent), and 0.04 µg/mL yeast transfer RNA. Aliquots should be stored at -20^0C or lower.

PCR Reaction Buffer: A 10-50 mM Tris-HCl solution (pH 8.3) containing 25 mM potassium chloride, 1.5 to 2.25 mM magnesium chloride, and 0.1 mg/mL gelatin. Aliquots should be stored at -20^0C or lower.

Isopropanol, stored at room temperature.

Ethanol, a 70-75% solution, stored at room temperature.

MuLV Reverse Transcriptase (4,000 U/mL), stored at -20^0C.

Dithiothreitol, 0.1 M solution. Aliquots should be stored at -20^0C or lower.

RNAsin (40,000 U/mL), or equivalent, stored at -20^0C.

Noniodet P-40, a 10% solution, stored at room temperature.

Taq DNA polymerase (2.5 units/mL), stored at -20^0C or lower.

Deoxynucleotide (dNTPs): A solution containing 25 mM each of deoxyguanosine (dGTP), deoxyadenosine (dATP), deoxycytosine (dCTP), deoxythymidine (dTTP). Aliquots should be stored at -20^0C or lower.

PCR primer "A'": A solution containing the oligomer
5'-TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "NE1'": A solution containing the oligomer
5'-CTTACTAACTTCTGTATGTCATTGACAGTCCAGCT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "B": A solution containing the oligomer
5'-GGATGGAAAGGATCACC-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "215WT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGGT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "215MUT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGAA-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

Water: Distilled grade or higher, stored at room temperature.

Wildtype Control DNA: A solution containing DNA from the HIV RT gene containing the wildtype (ACC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

Mutant Control DNA: A solution containing DNA from the HIV RT gene containing the mutant (TAC or TTC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

TBE Buffer: A solution containing 0.089M Tris borate (pH 8.3) and 0.01M EDTA, stored at room temperature.

Agarose: Molecular-biology grade, stored at room temperature.

Molecular Weight Marker: 100 base-pair marker (or equivalent) for electrophoresis, stored at 4^{0}C.

Gel Loading Buffer: A solution of 30% glycerol containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, or equivalent, stored at 4^{0}C.

IV. SUPPLIES AND EQUIPMENT

1.5 mL Siliconized Microcentrifuge Tubes
PCR Reaction Tubes and Caps
Perkin-Elmer GeneAmp PCR 9600 Thermal Cycler (or equivalent)
Pipettors (20 µL, 200 µL, and 1000 µL)
Aerosol-resistant Pipette Tips
Vortex Mixer
Water Bath, Incubator, or Heat Block for 56^{0}C (±2^{0}C) and 99^{0}C (±2^{0}C)
Speed-Vac, or equivalent (optional)
Agarose Electrophoresis Apparatus
Camera
Ultraviolet light source
High Speed Film
Latex Gloves
Lab Coat

V. PROCEDURE

A. Sample Preparation.

1. Method A.

a. Pipette 0.2 to 1.0 mL of test sample into a 1.5 mL microcentrifuge tube.

b. Centrifuge the sample for 30 min at 24,000 g or 10 min at 125,000 x g.

c. Discard the supernatant, add 400 µL of Solution D, and vortex.

d. Add 10 µL yeast transfer RNA and 26 µL of sodium acetate. Vortex.

e. Add 400 µL of phenol and 80 µL of chloroform:isoamyl alcohol. Vortex.

f. Place the samples on ice for 10 min.

g. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

h. Transfer the upper aqueous layer to a new 1.5 mL microcentrifuge tube, and appropriately discard the lower organic layer.

i. Add 450 µL of isopropanol to each sample, vortex, and hold at -20^0C for at least 1 hour.

j. Centrifuge the samples for 15 min at 12,000 x g at 4^0C.

k. Discard the supernatant, and add 1 mL of 70-75% ethanol.

l. Add 500 µL of 70-75% ethanol and vortex.

m. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

n. Discard the supernatant, and add 0.5 mL of 70-75% ethanol.

o. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

p. Discard the supernatant, and add 0.2 mL of 70-75% ethanol.

q. Centrifuge the samples for 15 min at 12,000 x g at 4^0C.

r. Discard the supernatant.

s. Air dry the pellet or use a Speed-Vac, just to dryness (do not over dry).

t. Resuspend the dried pellet on 25 µL of Viral Lysis Buffer.

2. Method B.

a. Pipette 0.2 to 1.0 mL of test sample into a 1.5 mL microcentrifuge tube.

b. Centrifuge the sample for 30 min at 24,000 g or 10 min at 125,000 x g.

c. Discard the supernatant, add 800 µL of Tri-Reagent, and vortex.

d. Hold at room temperature for 3 min.

e. Add 160 µL of chloroform and vortex.

f. Hold at room temperature for 3 min.

g. Centrifuge at 12,000 x g for 15 min at 4^0C.

h. Transfer the upper aqueous layer to a new 1.5 mL microcentrifuge tube.

i. Add 160 µL of chloroform to the remaining organic layer, and vortex.

j. Centrifuge the organic layer at 12,000 x g for 15 min at 4^0C.

k. Remove the upper aqueous phase and add it to the previously collected aqueous layer (step h).

l. Appropriately discard the remaining lower organic phase.

m. Add 400 µL of isopropanol to the aqueous phase and vortex.

n. Hold the samples at -20^0C overnight.

o. Centrifuge the samples at 12,000 g for 14 min at 4^0C.

p. Discard the supernatant.

q. Add 1 mL of 70-75% ethanol and vortex.

r. Discard the supernatant.

s. Air dry the pellet.

t. Resuspend the pellet in 25 µL of Viral Lysis Buffer.

B. Reverse Transcription.

1. For each sample to be tested, prepare the following reaction mix:

5 µL PCR Reaction Buffer
1 µL dNTPs
1 µL primer NE1'
2 µL reverse transcriptase
2 µL RNAsin
2 µL 0.1M DTT
3.4 µL 10% NP-40
10.4 µL water

2. Dispense 25 µL of reaction mix into PCR reaction tubes.

3. Add the 25 µL of test sample into a reaction tube.

4. Incubate the reactions for 10 min at 25^0C, 30 min at 42^0C, and 5 min at 95^0C.

C. First Round PCR.

1. For each sample to be tested, prepare the following reaction mix:
5 µL PCR Reaction Buffer
1 µL dNTPs
1 µL primer A'
0.5 µL Taq polymerase
42.5 µL water

2. Mix the reaction mix thoroughly.

3. Pipette 50 µL of the test sample into the reaction tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
35 cycles of:	94^0C for 30 sec
	55^0C for 30 sec
	72^0C for 60 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples can be stored at 4^0C for 24 hr or -20^0C or lower for longer periods.

C. Second Round PCR.

1. For each sample to be tested, prepare the following reaction mixtures:

Wildtype:	10 µL PCR Reaction Buffer
	1 µL dNTPs
	1 µL primer B
	2 µL primer 215WT
	0.5 µL Taq polymerase
	81.5 µL water

Mutant:	10 µL PCR Reaction Buffer
	1 µL dNTPs
	1 µL primer B
	2 µL primer 215MUT
	0.5 µL Taq polymerase
	81.5 µL water

2. Mix the reaction mixes thoroughly and pipette 95 µL of each reaction mix to separate PCR reaction tubes.

3. Pipette 5 µL of the first round PCR product into a wildtype reaction mix tube and another 5 µL into a mutant reaction mix tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
30 cycles of:	94^0C for 60 sec
	48^0C for 30 sec
	72^0C for 30 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples may be analyzed immediately or stored at 4^0C or lower.

D. Gel Electrophoresis.

1. Prepare a 3% agarose gel containing 0.5 µg/mL ethidium bromide in TBE.

2. Pipette 5 µL of Gel Loading Buffer into two microcentrifuge tubes.

3. Pipette 10-20 µL of each second-round PCR product into a tube and mix.

4. Load the samples into adjacent wells on the gel. Include 1 lane of 100 bp ladder per gel.

5. Run the gel in TBE for approximately 1 hr at 120V.

6. Remove the gel from the electrophoresis apparatus and photograph the gel under ultraviolet light.

VI. CALCULATIONS/DATA ANALYSIS

Samples which have a 210 base-pair product (band) in the wildtype reaction and do not have a 210 base-pair product in the mutant reaction are considered to have wildtype sequences at Codon 215. Samples which have a 210 base-pair product in the mutant reaction and do not have a 210 base-pair product in the wildtype reaction are considered to have mutant sequences at Codon 215. If a 210 base-pair product is detected in both reactions, the first round PCR product is diluted 1:1000, and 10 µL of the dilution is amplified in another second round PCR reaction. If a 210 base-pair product persists in the wildtype reaction but disappears from the mutant reaction, the sample is considered to have the wildtype sequence. If a 210 base-pair product persists in the mutant reaction but disappears from the wildtype reaction, the sample is considered to have the mutant sequence. If a 210 base-pair product persists in both wildtype and mutant reactions, the sample is considered to have a mixture of wildtype and mutant sequences at Codon 215.

VII. QUALITY CONTROL

For all amplifications, a reaction mix control consisting of reaction mix with water instead of test sample is required. The sample results for a given assay are invalid when a 210 base-pair band is detected in this control.

The second round PCR requires control reactions consisting of reaction mix with first-round PCR products of known wildtype and mutant viruses. These reactions will validate the ability of the wildtype and mutant reactions to accurately detect wildtype or mutant DNA in the test samples. The sample results for a given assay are invalid when either of these controls do not yield the appropriate 210 base-pair product.

VIII. PROCEDURE NOTES

A. Work areas in which the different PCR steps are performed should be geographically separated. At least, the room in which the second-round PCR product is analyzed should be separate from the areas in which reaction mixes and samples are prepared. A better

arrangement is to have a separate room for product analysis, sample preparation, and reaction mix preparation.

B. Good laboratory technique is essential to the proper performance of this assay. Extreme care should be taken to avoid the contamination of reagents with samples, controls, or PCR product and RNase in the sample preparation steps. Any reagent suspected of contamination should be discarded.

C. All pipettors, tips, reagents, and lab coats should be dedicated to and kept in only one PCR area (e.g., pipettors for loading gels cannot be used to prepare samples or reagents; lab coats worn during loading gels should not be worn into the reagent preparation area).

D. Pipette tips with aerosol barriers should be used during all steps.

E. PCR reaction trays (if used) should be soaked in bleach, rinsed with water, and dried before being used for another set of reactions.

F. Operators should pay close attention when transferring first-round PCR product into second-round reactions. Transferring an inadequate volume to one of the two reactions (wildtype or mutant), completely missing one of the two reactions, or confusing the identity of sample tubes can provide incorrect results without the mistake being obvious.

IX. LIMITATIONS

A. As with any PCR-based test, accurate detection of the Codon 215 mutation depends on extreme care being taken by laboratory technicians in handling reagents and performing the test, to ensure that any results obtained are derived from the patient's specimen and not from external sources.

B. Samples collected in heparin-containing tubes, vials, or equipment interfere with PCR reactions, which often results in no amplification. To ensure the greatest possibility of obtaining results, specimens should be collected in non-heparin-containing vessels.

C. The presence or absence of Codon 215 mutations cannot independently predict or determine the course of HIV infection. The results of this test should only be interpreted in conjunction with other laboratory and clinical data available to the clinician.

X. REFERENCES

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NEUTRALIZING ANTIBODY EVALUATION
INFECTIVITY REDUCTION ASSAY

I. PRINCIPLE

The HIV-1 neutralizing antibody assessment by the infectivity reduction assay estimates the reduction of the number of infectious units per milliliter (IUPML) in 1 mL of viral stock dilutions when treated with HIVIG or patient serum. This assay is performed in a 24-well plate and is based on the ACTG consensus plasma quantitative culture assay, using six 5-fold dilutions of viral isolate neutralized by HIVIG, serum or plasma. A 1:125 final concentration of HIVIG is added to each of the dilutions. When autologous serum is used, 1:20 or 1:40 dilution of serum is preferred. Each sample of viral dilution is cocultured with PHA-stimulated normal donor PBMC for 14 days. The supernatant for each individual well is assayed for HIV-1 p24 antigen as a determination of viral growth. The antigen results from each well can be evaluated by a software program to determine the IUPML in the presence of HIVIG, patient serum or normal human serum (control) according to the ACTG Virology Manual. The difference between the IUPML would be used to assess neutralizing capabilities of the test specimen. Based on preliminary studies, assay variation is expected to be 0.7 log.

II. SPECIMEN REQUIREMENTS

Viral stock of autologous or heterologous HIV-1 to be assayed. A pretitered viral stock is not necessary. A virus titration in the presence of normal human serum is included for each virus as part of the assay. (Please see "control set" in Assay Setup below.)

Patient serum/plasma. ACD or CPD-A1 tubes are recommended for plasma collection.

III. REAGENTS

RPMI-1640 with L-glutamine (2 mM) - Store a 4-8^{0}C and note manufacturer's outdate.

Fetal Bovine Serum - Store frozen at -20^{0}C and note manufacturer's outdate. When needed, rapidly that a bottle in a 37^{0}C water bath, then heat-inactivate in a 56^{0}C water bath for 30 minutes with occasional shaking. The level of water in the water bath should be as high as the level of serum in the bottle. Store at 4^{0}C after heat-inactivating for one month.

5% natural, delectinated IL-2 (Boehringer Mannheim, Cellular Products Inc., or Pharmacia). Store at -20^{0}C and note manufacturer's outdate.

0.001% DEAE-Dextran or 2 g/mL polybrene (optional).

Penicillin (100 units/mL)/streptomycin (100 g/mL) or gentamicin (50 g/mL).

Culture Medium - Prepare and store at 4-8^{0}C for up to one month.

RPMI-1640 with glutamine.
20% fetal bovine serum (heat-inactivated).
5% natural, delectinated IL-2 (Boehringer Mannheim, Cellular Products Inc., or Pharmacia).
0.001% DEAE-Dextran or 2 g/mL polybrene (optional).
Penicillin (100 units/mL)/streptomycin (100 g/mL) or gentamicin (50 g/mL).

PHA-stimulated Peripheral Blood Mononuclear Cells (PBMC)--see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC) in this manual.

NOTE: It is anticipated that the following reagents will be provided centrally by the Virology Quality Assurance (VQA) Laboratory.

Neutralizing Antibody (i.e.,, HIVIG monoclonal antibody).

Pooled Normal Human Sera (NHS).

Primary isolate, MN.

IV. EQUIPMENT AND SUPPLIES

Lab coat.
Gloves.
Laminar flow hood (Class 2 biosafety hood).
Micropipet(s) capable of delivering 20-1000 µL volumes.
Multichannel pipette capable of delivering 20-100 µL volumes.
Disposable sterile pipette tips suitable for the above pipettes (optional).
Disposable reagent reservoirs (optional).
12 x 75 mm sterile, polypropylene tubes.
Sterile 96-well plates (optional).
Sterile 24-well plates (optional).
5 mL polypropylene tubes.
Serological pipettes.
5 x 8 inch low-density polyethylene bags (Nalgene 6255-05088).
Cryovials.
CO_2 incubator (37$\pm$1^{0}C with humidity).
Centrifuge capable of speeds up to 400g and equipped O ring sealed safety cups.
Compound microscope.
Hemacytometer.
Automated cell counter (optional).

V. PROCEDURE

1. Prepare 2 sets of five-fold dilutions of undiluted virus stock (e.g., MN, primary isolate). One set of virus dilutions is the "test set" to which test antibody will be added. The other set of virus dilutions is the "control set" to which control serum will be added.

   Test Set:

   a. Place 1000 µL of undiluted virus into a tube labeled virus stock.

   b. Add 840 µL of culture medium to each of six 12 x 75 mm sterile tubes labeled A to F, or 140 µL into each of six wells if using a 96-well plate for the dilutions.

   c. To tube A, add 210 µL (35 µL for 96-well format) of the viral stock and mix thoroughly. Remove 210 µL (35 µL for 96-well format) from tube A and add to tube B. Repeat this process for a total of six dilutions. The resulting dilution scheme is 1:5 (tube A), 1:25 (tube B), 1:125 (tube C), 1:625 (tube D), 1:3125 (tube E), 1:15625 (tube F). Discard 210 TL (35 µL for 96-well format) from the last dilution (tube F).

   Control Set:

   a. Place 1000 µL of undiluted virus into a tube labeled virus stock.

   b. Add 840 µL of culture medium to each of eight 12 x 75 mm sterile tubes labeled A' to H' (or 35 µL for 96-well format).

   c. To tube A', add 210 µL (or 35 µL for 96-well format) of the viral stock and mix thoroughly. Remove 210 µL from tube A' and add to tube B'. Repeat this process for a total of eight dilutions. The resulting dilution scheme is 1:5 (tube A'), 1:25 (tube B'), 1:125 (tube C'), 1:625 (tube D'), 1:3125 (tube E'), 1:15625 (tube F'), 1:78125 (tube G'), 1:390625 (tube H'). Discard 210 µL (or 35 µL for 96-well format) from the last dilution (tube H').

2. To virus dilution tubes A-F in the "test set" add neutralizing antibody (e.g., HIVIG, monoclonal antibody) at a pre-determined dilution. In the case of HIVIG, add 6.7 µL of undiluted stock HIVIG to the 840 µL already in the tube, for a final dilution of 1:125 (or 1.1 µL to 140 µL if using 96-well format). Add the same concentrations of pooled normal human sera (NHS) to tubes C'-H' in the "control set". Incubate both sets of tubes for 1 hour at 37^0C.

3. Centrifuge 48 hours old PHA stimulated normal donor PBMC at 400 x g for 10 minutes at 20 to 24^0C, remove and discard supernatant. Resuspend cells in culture

medium and enumerate cells. Adjust sample with culture medium to a concentration of 1.25 x 10^6 PBMC/mL.

4. Set up 2 sets of PBMC sterile tubes (5 mL) or wells (24-well or 96-well plate, if you can centrifuge plates), corresponding to the "test set" of virus/HIVIG tubes and the "control test" of virus/NHS tubes.

   a. Add 3.2 mL of above PHA-stimulated PBMC to each tube or 1.6 mL of PBMC to each well (24-well plate) or 160 µL (96-well plate).

   b. Dispense 800 µL of appropriate virus/HIVIG or virus/NHS dilution to corresponding PBMC tubes, or 400 µL to each of two corresponding wells (24-well plate) or 40 µL to each well (96-well plate).

5. Place the tubes or plate into a 5% CO_2, humidified chamber or cover the plate with a 5 x 8 inch low-density polyethylene bag. Incubate overnight the samples at 37^0C. This is day 0.

6. On day 1, after overnight incubation, wash cells 2 times (400 x g for 10 minutes) with culture medium in tubes or wells. If using the well method, after the last wash resuspend in 2 mL (24-wells) or 200 µL (96-wells) of fresh culture medium and return to the corresponding well. If using the tube method, after the last wash resuspend in 4 mL of fresh plasma culture medium and return to the corresponding tube. Transfer cells from corresponding tubes to wells on a 24-well plate (tube A to wells A1, A2; tube B to wells B1, B2, etc.).

7. On day 7, remove one half of the medium (1 mL) or 100 µL if 96-well plates were used and save for future testing (save at -70^0C) and replace with 1 mL of fresh culture medium containing 0.5 x 10^6, 48-hour PHA-stimulated normal donor PBMC or 100 µL if using 96-well plates. Cultures are terminated on day 14, at which time 1.0 mL of medium is withdrawn and stored at -70^0C for subsequent HIV p24 antigen determination.

   For the labs using the 96-well format, the 100 µL harvested either on day 7 or day 14 will be put in 96-well plates and mixed with 100 µL of serum containing media. Cover the plates with sealer and freeze in -70^0C.

   Note: Day 7 culture supernatants should be tested if day 14 shows virus overgrowth (lack of any neutralization).

VI. CALCULATIONS

1. Wells are scored positive if p24 > 30 pg/mL. To calculate infectivity reduction, please enter p24 values (raw data) into virology software to calculate IUPM.

Obtain IUPM for the "control test" (NHS) and for the "test set" (HIVIG). Express infectivity reduction as the *ratio* of IUPM (control) to IUPM (test).

VII. RESULTS

A ten fold reduction equals 90% neutralization.

Note: In preparation for implementing this assay in clinical studies, more discussion will take place regarding neutralization data capture and analysis. Efforts will be made to incorporate the principles of the ID_{50} program developed by John Spouge, M.D. This program calculates virus infectious doses by fitting a model to the data. It obtains log ID50 for the "control set" (NHS) and for the "test set" (HIVIG). Infectivity reduction is expressed as the *difference* between logID_{50} (control) and logID50 (test). One log reduction equals 90% neutralization. For more information about this program, please call Dr. Spouge at 301-496-2475.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 P24 ANTIGEN
Dupont *Alliance*™
HIV-1 p24 ELISA

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The Dupont HIV-1 p24 Antigen Assay is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Dupont HIV-1 p24 Antigen Assay uses a highly specific mouse monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma, serum or tissue culture medium and lysis buffer are added to a coated well and incubated. If present, the viral antigens bind to the monoclonal antibody on the microtiter well. Following a wash step, biotinylated polyclonal antibody to HIV-1 p24 is added to the well and, during incubation, binds to any HIV-1 p24 antigen bound to the well. Following another wash step, streptavidin-horseradish peroxidase conjugate is added which complexes with biotinylated antibodies. In a final step, a substrate reagent containing orthophenylenediamine-HCL (OPD) is added which reacts with complexed peroxidase to form a yellow color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color development is directly proportional to the amount of p24 antigen in the plasma, serum or tissue culture medium. The quantity of HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of a known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum, tissue culture supernatant, or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C to -85^{0}C for long-term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in the Dupont HIV-1 p24 ELISA, 96 (PN 6604534) or 2400 (PN 6607051) kits are:

1. HIV-1 p24 Antibody-coated Microtiter Strips. Store at 2-8^{0}C. Note manufacturer's outdate.

   a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

   b. The plate consists of 12 removable strips of 8 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 12 strips are needed, place uncoated strips in the remaining positions.

   c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Detector Antibody (Rabbit polyclonal anti-p24 antibody). Store at 2-8^{0}C. Note manufacturer's outdate.

3. Streptavidin-HRP Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

4. Streptavidin-HRP Concentrate. Store at 2-8^{0}C. Note manufacturer's outdate.

   a. Within 15 minutes prior to use, prepare Streptavidin-HRP by making a 1:100 dilution of Streptavidin-HRP Concentrate with Streptavidin-HRP Diluent. To prepare the working concentration for a <u>complete 96 well plate</u> add 22 µL of the Streptavidin-HRP Concentrate to 22 mL of Streptavidin-HRP Diluent.

   b. If a partial plate is used, prepare enough Streptavidin-HRP working concentration as shown below:

No of Strips	SA-Buffer (mL)	SA-HRPO (µL)
4	4.0	4
6	6.0	6
12	12.0	12
24	22.0	22

c. Discard unused portion at the end of the day.

5. Substrate Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

6. OPD Tablets.

a. Within 15 minutes of use prepare sufficient OPD Substrate Solution. With non-metallic forceps or the equivalent, add 1 OPD Tablet to 11 mL of Substrate Diluent for each plate or partial plate assayed.

b. Vortex vigorously to assure complete dissolution.

c. Protect from light. The OPD substrate solution should be colorless to pale yellow. A yellow-orange color indicates that the reagent is contaminated and must be discarded.

d. Discard unused portion at the end of the day.

7. 5% Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

8. Plate Wash Concentrate 20X. Store at 2-8^{0}C. Note manufacturer's outdate.

a. Dilute Plate Wash Concentrate 20X by adding 1 part concentrate to 19 parts distilled, deionized water (i.e., 200 mL Wash Concentrate / 1800 mL dH_2O).

b. Crystals may form in the Plate Wash Concentrate 20X if refrigerated. These should be redissolved by gently warming prior to use.

c. Approximately 1000 mL of diluted (1X) wash buffer is needed per plate assayed. More or less may be needed depending on the type of washer used. Diluted (1X) wash buffer should be prepared fresh prior to use. However, once prepared the diluted (1X) wash buffer has a 1 week expiration date.

9. Stop Solution (4N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100, or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.
2) Mix well and use.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10-1000 µL volumes
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Strip holder reaction plate
Serological pipettes
Vortex mixer
Centrifuge
Incubator without CO_2 capable of maintaining 37^{0}C +/- 2^{0}C
Timer capable of measuring times up to 60 minutes
Graduated cylinders and beakers
ELISA microtiter plate washer with waste trap and vacuum source
ELISA microplate plate reader capable of measuring absorbance at 490 or 492 nm with reference at 600 nm

V. PROCEDURE

1. Bring all reagents and samples to room temperature.

2. Create an EIA template in the virology data-management software (see software manual).

3. Position the required number of microtiter strips in the strip holder reaction plate (8 wells per strip). If fewer than 12 strips are needed, use uncoated strip(s) in the remaining positions when using a 96 well plate washer.

4. Add 20 µL of Triton-X to each test well of the coated microtiter plate.

5. Add 200 µL of each VQA SQC concentration and each specimen to the coated microtiter plate according to the template. Cover the plate using an adhesive plate cover.

6. Incubate at 37^0C for 2 hours.

7. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash five (5) more times for a total of 6 washes. After the final wash step, grasp the plate <u>firmly</u> along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

   Important: The time between the wash step and the next reagent must be less than five (5) minutes.

8. Add 100 µL of Detector Antibody to all testing wells, <u>except</u> the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 1 hour $\pm$ 5 minutes.

9. Wash as described above.

10. Add 100 µL of Streptavidin-HRPO Working Dilution to all testing wells, <u>except</u> the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at room temperature (15^0C-30^0C) for 30 $\pm$ 5 minutes.

11. Wash as described above.

12. Add 100 µL of freshly prepared OPD-Substrate Solution to all wells. Cover the plate using a new adhesive plate cover. Incubate at room temperature, <u>in the dark</u>, (15-30^0C) for 30 + 5 minutes.

13. Add 100 µL of Stop Solution to all wells.

14. Read the absorbance at 490 or 492 nm, blanking the plate reader on air, (Consult the plate reader Instruction Manual for specific directions) within 15 minutes after

adding Stop Solution. Readings must be taken with a reference filter at >600 nm. Be sure the bottom of the plate is clean and dry prior to reading.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained from the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and are reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

Use only reagents from the same kit lot. Do not interchange vials or bottle caps and stoppers.

Plate washing may be automated, semi-automated or manual, but must be carried out with care to ensure optimal performance of the assay. It is recommended that six remove/fill cycles be performed.

IX. REFERENCES

Dupont HIV-1 p24 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 p24 ANTIGEN
Dupont *Alliance*™
HIV-1 p24 ELISA
Immune Complex Dissociation

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The ICD HIV-1 antigen assay is a modification of the standard HIV-1 antigen assay. Circulating HIV-1 antigen binds to native HIV-1 antibody to form an immune complex that is hidden from detection by the standard assay. The dissociation of the immune complex, accomplished by pH and heat, allows the p24 antigen to become detectable by the routine assay.

The Dupont HIV-1 p24 Antigen Assay is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Dupont HIV-1 p24 Antigen Assay uses a highly specific mouse monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma or serum and lysis buffer are added to a coated well and incubated. If present, the viral antigens bind to the monoclonal antibody on the microtiter well. Following a wash step, biotinylated polyclonal antibody to HIV-1 p24 is added to the well and, during incubation, binds to any HIV-1 p24 antigen bound to the well. Following another wash step, streptavidin-horseradish peroxidase conjugate is added which complexes with biotinylated antibodies. In a final step, a substrate reagent containing orthophenylenediamine-HCL (OPD) is added which reacts with complexed peroxidase to form a yellow color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color development is directly proportional to the amount of p24 antigen in the plasma or serum. The quantity of HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of a known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the

specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C to -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in the Dupont HIV-1 p24 ELISA, 96 (PN 6604534) or 2400 (PN 6607051) kits are:

1. HIV-1 p24 Antibody-coated Microtiter Strips. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

    b. The plate consists of 12 removable strips of 8 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 12 strips are needed, place uncoated strips in the remaining positions.

    c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Glycine Reagent. Store at 2-8^{0}C. Note the manufacturer's outdate.

3. Tris Reagent. Store at 2-8^{0}C. Note the manufacturer's outdate.

4. Neutral Buffer:

a. Prepare 400 µL Neutral Buffer by mixing together equal volumes of Glycine Reagent (200 µL) and Tris Reagent (200 µL).

b. Discard unused portion at the end of the day.

5. Detector Antibody (Rabbit polyclonal anti-p24 antibody). Store at 2-8^{0}C. Note manufacturer's outdate.

6. Streptavidin-HRP Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

7. Streptavidin-HRP Concentrate. Store at 2-8^{0}C. Note manufacturer's outdate.

a. Within 15 minutes prior to use, prepare Streptavidin-HRP by making a 1:100 dilution of Streptavidin-HRP Concentrate with Streptavidin-HRP Diluent. To prepare the working concentration for a complete 96 well plate add 22 µL of the Streptavidin-HRP Concentrate to 22 mL of Streptavidin-HRP Diluent.

b. If a partial plate is used, prepare enough Streptavidin-HRP working concentration as shown below:

No of Strips	**SA-Buffer (mL)**	**SA-HRPO (µL)**
4	4.0	4
6	6.0	6
12	12.0	12
24	22.0	22

c. Discard unused portion at the end of the day.

8. Substrate Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

9. OPD Tablets.

a. Within 15 minutes of use prepare sufficient OPD Substrate Solution. With non-metallic forceps or the equivalent, add 1 OPD Tablet to 11 mL of Substrate Diluent for each plate or partial plate assayed.

b. Vortex vigorously to assure complete dissolution.

c. Protect from light. The OPD substrate solution should be colorless to pale yellow. A yellow-orange color indicates that the reagent is contaminated and must be discarded.

d. Discard unused portion at the end of the day.

10. 5% Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

11. Plate Wash Concentrate 20X. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Dilute Plate Wash Concentrate 20X by adding 1 part concentrate to 19 parts distilled, deionized water (i.e., 200 mL Wash Concentrate / 1800 mL dH_2O).

    b. Crystals may form in the Plate Wash Concentrate 20X if refrigerated. These should be redissolved by gently warming prior to use.

    c. Approximately 1000 mL of diluted (1X) wash buffer is needed per plate assayed. More or less may be needed depending on the type of washer used. Diluted (1X) wash buffer should be prepared FRESH prior to use. However, once prepared the diluted (1X) wash buffer has a 1 week expiration date.

12. Stop Solution (4N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

    a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

        1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.

        2) Mix well and use.

    b. ICD p24 Positive Control. Once reconstituted, store at 2 -8^{0}C for up to 2 weeks.

        1) Reconstitute the lyophilized ICD positive control with 2.0 mL of dH_2O.

        2) Gently mix by inversion and allow 15 minutes for the reagent to go into solution.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10-1000 µL volumes
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volume
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Uncoated microtiter plate
Strip holder reaction plate
Serological pipettes
Centrifuge
Vortex mixer
Incubator without CO_2 capable of maintaining 37^0C +/- 2^0C
Timer capable of measuring times up to 60 minutes
Graduated cylinders and beakers
ELISA microtiter plate washer with waste trap and vacuum source
ELISA microtiter plate reader capable of measuring absorbance at 490 or 492 nm with reference at 600 nm

V. PROCEDURE

A. ICD Sample Preparation

1. Bring all reagents and samples to room temperature.

2. Create an EIA template in the virology data-management software (see software manual).

3. Dispense 20 µL of 5% Triton-X into each test well of an uncoated microtiter plate.

4. Dispense 90 µL of each VQA SQC concentration, the ICD Positive Control and each specimen into the uncoated microtiter plate according to the template. The ICD Positive control will be treated with ICD Glycine Reagent or Neutral Buffer as described in step 5., resulting in Treated ICD Positive Control, T100, and Untreated ICD Positive Control, U100.

5. Dispense 90 µL of Glycine Reagent into each well except the U100 ICD Positive Control, with a multichannel pipettor. To the U100 ICD Positive Control, add 90 µL Neutral Buffer. Mix the reagents in the wells by filling and dispensing five times. Avoid foaming. Change tips before proceeding to each new row.

6. Carefully seal the plate with an adhesive plate cover. Place the plate in the incubation bag and seal the bag. Incubate at $37^0C \pm 1^0C$ for 1 hour $\pm$ 5 minutes.

7. Remove the plate from the incubator and remove the plate cover. Dispense 90 µL of Tris Reagent to each of the wells except the U100 (ICD Positive control in which you again add 90 µL of Neutral Buffer) with a multichannel pipette. Mix the reagents in the wells by filling and dispensing five times. Avoid foaming by not fully dispensing pipette tip contents when mixing. Change tips before preceding to the next row.

8. Incubate the plate at room temperature (15 -30^{0}C) for 20 minutes.

9. Using a multichannel pipette transfer 200 µL of all wells to the corresponding wells of the Dupont HIV-1 p24 antigen coated microtiter strip reaction plate.

10. Carefully seal the plate with an adhesive plate cover. Incubate the plate at 37^{0}C $\pm$1^{0}C for 2 hours.

B. HIV-1 p24 Antigen Assay

1. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash five (5) more times for a total of 6 washes. After the final wash step, grasp the plate firmly along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

2. Add 100 µL of Detector Antibody to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^{0}C $\pm$ 1 for 1 hour $\pm$ 5 minutes.

3. Wash as described above.

4. Add 100 µL of Streptavidin-HRPO Working Dilution to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at room temperature (15^{0}C-30^{0}C), for 30 $\pm$ 5 minutes.

5. Wash as described above.

6. Add 100 µL of freshly prepared OPD-Substrate Solution to all wells. Cover the plate using a new adhesive plate cover. Incubate at room temperature (15-30^{0}C), in the dark, for 30 $\pm$ 5 minutes.

7. Add 100 µL of Stop Solution to all wells.

8. Read the absorbance at 490 or 492 nm, blanking the plate reader on air, (Consult the plate reader Instruction Manual for specific directions) within 15 minutes after

adding Stop Solution. Readings must be taken with a reference filter at > 600 nm. Be sure the bottom of the plate is clean and dry prior to reading.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained from the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and are reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

Use only reagents from the same kit lot. Do not interchange vials or bottle caps and stoppers.

Plate washing may be automated, semi-automated or manual, but must be carried out with care to ensure optimal performance of the assay. It is recommended that six remove/fill cycles be performed.

IX. REFERENCES

Dupont HIV-1 p24 Antigen Assay package insert and all references within.

Dupont ICD HIV-1 p24 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)
Vironostika® HIV-1 Antigen Microelisa System
Organon Teknika

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The Vironostika® HIV-1 p24 Antigen Microelisa System is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Vironostika HIV-1 assay uses a murine monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma, serum or tissue culture medium and lysis buffer are added to a coated well and incubated. If present, the viral antigens bind to the monoclonal antibody on the microtiter well. Subsequently, anti-HIV-1 (human) conjugate labeled with HRP is added. The labeled antibody binds to the solid phase antibody/antigen complexes previously formed. In a final step, a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxide is added which reacts with complexed peroxidase to form a blue color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color development is directly proportional to the amount of uncomplexed p24 antigen in the plasma, serum or tissue culture media. The quantity of free HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum, tissue culture supernatant or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. The Vironostika® HIV-1 Antigen Microelisa Assay, 192 (PN 59464) kits include the following reagents:.

1. HIV-1 p24 Antibody-coated Microelisa Strips. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

    b. The plate consists of 8 removable strips of 12 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 8 strips are needed, place uncoated strips in the remaining positions.

    c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Anti-HIV-1 Conjugate (human)-Horseradish peroxidase-labeled anti-HIV-1. Store at 2-8^{0}C. Note manufacturer's outdate.

3. Disruption Buffer. Store at 2-8^{0}C. Note manufacturer's outdate.

4. TMB Solution. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Within 10 minutes of use prepare the TMB-Substrate Solution by adding 1 mL of TMB Substrate to 1 mL of Peroxidase Solution for each Microelisa Strip that will be used.

5. Peroxide Solution. Store at 2-8^{0}C. Note manufacturer's outdate.

6. Phosphate Buffer Concentrate. Store at 2-8^{0}C. Note manufacturer's outdate.

a. Prepare at least 50 mL of Phosphate Buffer working solution for each Microelisa Strip used. Note: Approximately 700 mL of working solution is required for a complete 96 well plate.

b. Dilute the Phosphate Buffer Concentration 1:25 with distilled water.

c. Discard any unused reagent at the end of the day.

7. Stop Solution (2N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control - human serum nonreactive for HIV-1 antibody. Note manufacturer's outdate.

9. Positive Control - human HIV-1 antigen containing 160 pg/mL p24 core antigen (inactivated). Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.
2) Mix well and use.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Microelisa Strip Holder
Disposable vials
Serological pipettes
Incubator without CO_2 capable of maintaining 37^{0}C +/- 2^{0}C

Centrifuge
Timer capable of measuring times up to 60 minutes
Graduated cylinders and beakers
ELISA microtiter plate washer with waste trap and vacuum source
ELISA microtiter plate reader capable of measuring absorbance at 450 nm with reference at 630 nm

V. PROCEDURE

A. Plate Set-up

1. Bring all reagents and samples to room temperature.

2. Create an EIA template in the virology data-management software (see software manual).

3. Position the required number of microtiter strips in the strip holder reaction plate (12 wells per strip). If fewer than 8 strips are needed, use uncoated strip(s) in the remaining positions when using a 96 well plate washer.

4. Add 25 µL of Disruption Buffer to each test well of the coated microtiter plate.

5. Add 100 µL of each VQA SQC concentration and each specimen to the coated microtiter plate according to the template. Cover the plate using an adhesive plate cover.

6. Incubate at 37^0C for 1 hour $\pm$ 2 minutes.

7. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash three (3) more times for a total of 4 washes. After the final wash step, grasp the plate firmly along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

   Important: The time between the wash step and the next reagent must be less than five (5) minutes.

8. Add 100 µL of Anti-HIV-1 Conjugate to all testing wells (prepared during the last 10 minutes of incubation described above). Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 1 hour $\pm$ 2 minutes.

9. Wash as described above.

10. Add 100 µL of TMB Substrate to all testing wells. Incubate at room temperature (15^0C -30^0C) for 30 $\pm$ 2 minutes. Do not cover plate.

11. Add 100 µL of Stop Solution to all wells and mix by tapping.

    Important: Add Stop Solution to the wells in the same sequence and at the same rate of speed that the TMB Substrate was added.

12. Read absorbance at 450 nm (use 630 nm reference filter for dual wavelength instruments) within 15 minutes of adding Stop Solution to the wells. Blank the reader on air prior to reading.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

All pipetting steps should be performed with the utmost care and accuracy. Cross contamination between reagents and specimens will invalidate results. Use micropipettes for quantitative delivery of samples and reagents.

Use only reagents from the same kit lot.

To avoid contamination, do not touch the top of the Microelisa tips or the edge of the wells with fingers or pipette tips.

The aspiration/wash system should be flushed with copious amounts of water upon completion of the final wash of the assay.

Incomplete washing will adversely affect the test outcome. Phosphate Buffer must be at room temperature before use.

IX. REFERENCES

Vironostika® HIV-1 Antigen Microelisa System Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 p24 ANTIGEN
Coulter HIV-1 p24 Antigen Assay
(Murine Monoclonal)

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The Coulter HIV-1 p24 Antigen Assay is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Coulter HIV-1 p24 Antigen Assay uses a murine monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma, serum or tissue culture media and lysis buffer are added to a coated well and incubated. If present, the virus particles binds to the monoclonal antibody to the microtiter well. Following a wash step, biotinylated human anti-HIV-1 IgG is added to the well and, during incubation, binds to any HIV-1 p24 antigen bound to the well. Following another wash step, streptavidin-horseradish peroxidase is added which complexes with biotinylated antibodies. In a final step, a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxide is added which reacts with complexed peroxidase to form a blue color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color development is directly proportional to the amount of uncomplexed p24 antigen in the plasma, serum or tissue culture media. The quantity of free HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum, tissue culture supernatant, or plasma collected in acid-citrate-dextrose, (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long term storage.

Remove the serum from the clot or plasma from the red cells as soon as possible to avoid hemolysis.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in Coulter HIV-1 p24 Antigen Assay Kit, 96 (PN 6604534) or 2400 (PN 6607051), include the following:

1. HIV-1 p24 Antibody-coated Microtiter Strips. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

    b. The plate consists of 12 removable strips of 8 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 12 strips are needed, place uncoated strips in the remaining positions.

    c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Anti-HIV (human)-Biotin Reagent. Store at 2-8^{0}C. Note manufacturer's outdate. The reconstituted reagent is stable for 2 months. Bring to room temperature (15-30^{0}C) prior to assay.

    a. Add 21 mL of distilled water to the Biotin Reagent vial and recap the vial.

    b. Gently invert vial to mix contents. Allow 5 minutes for the contents to dissolve.

3. Normal Human Serum (NHS). Store at 2-8^{0}C. Note manufacturer's outdate.

4. SA-Buffer (Tris buffer for SA-HRPO). Store at 2-8^{0}C. Note manufacturer's outdate.

5. SA-HRPO (Streptavidin conjugated to horseradish peroxidase). Store at 2-8^{0}C. Note manufacturer's outdate. Prepare SA-HRPO Working Dilution as follows:

   a. Within 15 minutes prior to use, prepare the SA-HRPO Working Dilution. For a complete 96 well plate, add 21 µL of SA-HRPO reagent to 21 mL of SA-HRPO Buffer. Mix well and use.

   b. If a partial plate is used, prepare enough SA-HRPO Working Dilution as shown below:

No of Tests	SA Buffer (mL)	SA-HRPO (µL)
24	5.0	5
48	10.0	10
72	15.0	15

   c. Discard unused portion at the end of the day.

6. TMB Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

7. TMB Reagent in Dimethyl sulfoxide. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare TMB-substrate Solution as follows:

   a. Within 15 minutes prior to use prepare the TMB-Substrate Solution. For a complete 96 well plate add 21 mL of the TMB Diluent into a clean disposable plastic container and add 210 µL of TMB Reagent. Mix well and use.

   b. If a partial plate is used, prepare enough TMB-Substrate as follows:

No of Tests	TMB Diluent (mL)	TMB Reagent (µL)
24	5.0	50
48	10.0	100
72	15.0	150

   c. Discard unused portion at the end of the day.

   Note: TMB-Substrate Solution should appear colorless and, when combined with CSR-1 Solution, should have an absorbance value less than 0.050 at 450 nm 450/570 nm when compared wit a distilled water blank.

8. Lysis Buffer. Store at 2-8^{0}C. Note manufacturer's outdate.

9. Wash Buffer. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare Working Wash Buffer as follows:

    a. To prepare 700 mL of Wash Buffer, dilute 35 mL of 20X Wash Buffer with 665 mL of distilled water.

    b. Discard any unused portion at the end of the day.

10. Coulter Stop Reagent-1 (CSR-1) (4N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

    a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

        1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.
        2) Mix well and use.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volume
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Uncoated 96 well microtiter plate
Incubator without CO_2 capable of maintaining 37^{0}C +/- 1^{0}C
Timer capable of measuring times up to 60 minutes
Centrifuge
Graduated cylinders and beakers
Serological pipettes
ELISA microtiter plate washer with waste trap and vacuum source

ELISA microtiter plate reader capable of measuring absorbance at 450 nm with reference at 570 nm

V. PROCEDURE

A. Plate Set-up

1. Bring all reagents and samples to room temperature.

2. Create an EIA template in the virology data-management software (see software manual).

3. Position the required number of microtiter strips in the strip holder reaction plate (8 wells per strip). If fewer than 12 strips are needed, use uncoated strip(s) in the remaining positions when using a 96 well plate washer.

4. Add 20 µL of Lysis Buffer to each test well of the coated microtiter plate.

5. Add 200 µL of each VQA SQC concentration and each specimen to the coated microtiter plate according to the template. Cover the plate using an adhesive plate cover.

6. Incubate at 37^0C for 1 hour $\pm$ 5 minutes.

7. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash five (5) more times for a total of 6 washes. After the final wash step, grasp the plate firmly along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

Important: The time between the wash step and the next reagent must be less than five (5) minutes.

8. Add 200 µL of reconstituted Biotin Reagent to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 1 hour $\pm$ 5 minutes.

9. Wash as described above.

10. Add 200 µL of SA-HRPO Working Dilution to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C ± 2 for 30 ± 2 minutes.

11. Wash as described above.

12. Add 200 µL of TMB-Substrate Solution to all wells. Cover the plate using a new adhesive plate cover. Incubate at room temperature (15-30^0C) for 30 ± 2 minutes.

13. Add 50 µL of CSR-1 to all wells.

Important: Add CSR-1 to the wells in the same sequence and at the same rate of speed that the TMB-Substrate Solution was added.

14. Read absorbance at 450 nm (reference at 570 nm if dual wavelength Instrument is available) within 30 minutes of adding CSR-1 to the wells.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory s standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

The incubation at 37^0C is critical. If the temperature goes above 38^0C, coagulation of the samples may occur.

If a sample gels completely and the well still contains visible coagulated serum proteins after washing, the results should be considered invalid and the sample retested.

IX. REFERENCES

Coulter HIV-1 p24 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 p24 ANTIGEN
Coulter HIV-1 p24 Antigen Assay
(Murine Monoclonal)
Immune Complex Dissociation

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The ICD HIV-1 antigen assay is a modification of the standard HIV-1 antigen assay. Circulating HIV-1 antigen binds to native HIV-1 antibody to form an immune complex and that is hidden from detection by the standard assay. The dissociation of the immune complex, accomplished by pH and heat, allows the p24 antigen to become detectable by the routine assay.

The Coulter HIV-1 p24 Antigen Assay is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Coulter HIV-1 p24 Antigen Assay uses a murine monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma or serum and lysis buffer are added to a coated well and incubated. If present, the viral antigen binds to the monoclonal antibody to the microtiter well. Following a wash step, biotinylated human anti-HIV-1 IgG is added to the well and, during incubation, binds to any HIV-1 p24 antigen bound to the well. Following another wash step, streptavidin-horseradish peroxidase is added which complexes with biotinylated antibodies. In a final step, a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxide is added which reacts with complexed peroxidase to form a blue color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color is directly proportional to the amount of uncomplexed p24 antigen in the plasma or serum. The quantity of HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standards.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose, (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as

possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in Coulter HIV-1 p24 Antigen Assay Kit, 96 (PN 6604534) or 2400 (PN 6607051), include the following:

1. HIV-1 p24 Antibody-coated Microtiter Srtips. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

    b. The plate consists of 12 removable strips of 8 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 12 strips are needed, place uncoated strips in the remaining positions.

    c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Anti-HIV (human)-Biotin Reagent. Store at 2-8^{0}C. Note manufacturer's outdate. The reconstituted reagent is stable for 2 months. Bring to room temperature (15-30^{0}C) prior to assay.

    a. Add 21 mL of distilled water to the Biotin Reagent vial and recap the vial.

    b. Gently invert vial to mix contents. Allow 5 minutes for the contents to dissolve.

3. Normal Human Serum (NHS). Store at 2-8^{0}C. Note manufacturer's outdate.

4. SA-Buffer (Tris buffer for SA-HRPO). Store at 2-8^{0}C. Note manufacturer's outdate.

5. SA-HRPO (Streptavidin conjugated to horseradish peroxidase). Store at 2-8^{0}C. Note manufacturer's outdate. Prepare SA-HRPO Working Dilution as follows:

   a. Within 15 minutes prior to use prepare the SA-HRPO Working Dilution. For a complete 96 well plate, add 21 µL of SA-HRPO reagent to 21 mL of SA-HRPO Buffer. Mix well and use.

   b. If a partial plate is used, prepare enough SA-HRPO Working Dilution as shown below:

No of Tests	SA Buffer (mL)	SA-HRPO (µL)
24	5.0	5
48	10.0	10
72	15.0	15

   c. Discard unused portion at the end of the day.

6. TMB Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

   TMB Reagent in dimethyl sulfoxide. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare TMB-substrate Solution as follows:

   a. Within 15 minutes prior to use prepare the TMB-Substrate Solution. For a complete 96 well plate add 21 mL of the TMB Diluent into a clean, disposable plastic container and add 210 µL of TMB Reagent. Mix well and use.

   b. If a partial plate is used, prepare enough TMB-Substrate as follows:

No of Tests	TMB Diluent (mL)	TMB Reagent (µL)
24	5.0	50
48	10.0	100
72	15.0	150

   c. Discard unused portion at the end of the day.

   Note: TMB-Substrate Solution should appear colorless and, when combined with CSR-1 Solution, should have an absorbance value less than 0.050 at 450 nm 450/570 nm when compared wit a distilled water blank.

8. Lysis Buffer. Store at 2-8^{0}C. Note manufacturer's outdate.

9. Wash Buffer. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare Working Wash Buffer as follows:

   a. To prepare 700 mL of Wash Buffer, dilute 35 mL of 20X Wash Buffer with 665 mL of distilled water.

   b. Discard any unused portion at the end of the day.

10. Coulter Stop Reagent-1 (CSR-1) (4N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

B. Reagents included in Coulter ICD-Prep Kit (PN 6604709) include:

1. Glycine Reagent. Store at 2-8^{0}C. Note manufacturer's outdate.

2. Tris Reagent. Store at 2-8^{0}C. Note manufacturer's outdate.

3. Incubation bags.

C. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

   a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

      1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.
      2) Mix well and use.

   b. ICD p24 Positive Control. Once reconstituted, store at 2 -8^{0}C for up to 2 weeks.

      1) Reconstitute the lyophilized ICD positive control with 2.0 mL of dH_2O.
      2) Gently mix by inversion and allow 15 minutes for the reagent to go into solution.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volume.
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Uncoated 96 well microtiter plate
Strip holder reaction plate
Incubator without CO_2 capable of maintaining 37^0C +/- 1^0C
Timer capable of measuring times up to 60 minutes
Centrifuge
Graduated cylinders and beakers
Serological pipettes
ELISA microtiter plate washer with waste trap and vacuum source
ELISA microtiter plate reader capable of measuring absorbance at 450 nm with reference at 570 nm

V. PROCEDURE

A. ICD Sample Preparation

1. Bring all reagents and samples to room temperature.

2. Create an ICD EIA template in the virology data-management software (see software manual)

3. Dispense 30 µL of Lysis Buffer to each well that will receive either SQC, V100, ICD p24 Positive Control, or patient specimen as noted on the template. The ICD specimen pretreatment is set up using an uncoated microtiter plate!

4. Dispense 100 µL of SQC, ICD Positive Control or patient specimens as noted on the template. The ICD Positive control will be treated with Glycine Reagent or Neutral Buffer) as described in step 5., resulting in Treated ICD Positive Control, T100, and Untreated ICD Positive Control, U100.

5. Dispense 100 µL of Glycine Reagent to each of the wells except the U100 (ICD Positive control in which you add 100 µL of Neutral Buffer) with a multichannel pipette. Mix the reagents in the wells by filling and dispensing sample and Glycine reagent five times. Avoid foaming by not fully dispensing pipette tip contents when mixing. Change tips before preceding to the next strip.

6. Carefully seal the plate with an adhesive plate cover. Place it in the incubation bag and seal the bag. Incubate the plate at 37^0C for 90 mins.

7. Remove the plate from the incubator and remove the plate cover. Dispense 100 µL of Tris Reagent to each of the wells except the U100 (ICD Positive control in which you again add 100 µL of Neutral Buffer) with a multichannel pipette. Mix the reagents in the wells by filling and dispensing sample and Glycine reagent five times. Avoid foaming by not fully dispensing pipette tip contents when mixing. Change tips before preceding to the next strip. (Note: Samples may now sit for up to one hour before proceeding to the next step, if desired.)

8. Position the required number of HIV-1 p24 Antibody-coated Microtiter Strips in the strip holder reaction plate (8 wells per strip). If fewer than 12 strips are needed, use uncoated strip(s) in the remaining positions when using a 96 well plate washer. Using a multichannel pipette transfer 200 µL of all wells to the corresponding wells of the coated microtiter strip reaction plate.

9. Carefully seal the plate with an adhesive plate cover. Place it in the incubation bag and seal the bag. Incubate the plate at 37^0C overnight (18-24 hrs.).

B. HIV-1 p24 Antigen Assay

1. After overnight incubation, remove the reaction plate from the incubation bag and carefully remove the plate cover and discard.

2. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash five (5) more times for a total of 6 washes. After the final wash step, grasp the plate firmly along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

Important: The time between the wash step and the next reagent must be less than five (5) minutes.

3. Add 200 µL of reconstituted Biotin Reagent to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 1 hour $\pm$ 5 minutes.

4. Wash as described above.

5. Add 200 µL of SA-HRPO Working Dilution to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 30 $\pm$ 2 minutes.

6. Wash as above.

7. Add 200 µL of TMB-Substrate Solution to all wells. Cover the plate using a new adhesive plate over. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

8. Add 50 µL of CSR-1 to all wells.

Important: Add CSR-1 to the wells in the same sequence and at the same rate of speed that the TMB-Substrate Solution was added.

9. Read absorbance at 450 nm (reference at 570 nm if dual wavelength is available) within 30 minutes of adding CSR-1 to the wells.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and the Treated and Untreated ICD Positive Control and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

The incubation at 37^{0}C during the ICD Sample Preparation step is critical. If the temperature goes above 38^{0}C, coagulation of the samples may occur.

Occasionally, samples will produce a small amount of insoluble material after the overnight incubation. Aspiration of all wells before placing the plate in the washer will eliminate clogging of the plate washer.

If a sample gels completely and the well still contains visible coagulated serum proteins after washing, the results should be considered invalid and the sample retested.

IX. REFERENCES

Coulter HIV-1 p24 Antigen Assay package insert and all references within.

Coulter ICD-Prep Kit package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Monoclonal
Direct Serum Assay

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The Abbott HIVAG-1 Monoclonal Assay is an enzyme immunoassay (EIA) developed for detection uncomplexed HIV-1 p24 antigen. The Abbott HIVAG-1 Monoclonal Assay is a "sandwich" solid phase immunoassay that uses a polystyrene bead coated with human monoclonal antibody (Ab) to HIV-1 p24. If present, the viral antigen binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the beads, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the bead. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of known a HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C to -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. The following reagents are included in the Abbott HIVAG-1 Monoclonal Kit. Kit reagents may be used at room temperature or cold unless otherwise stated in reagent preparation.

1. HIV-1 (Human) Monoclonal Antibody-coated Beads. Store at 2-8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly for storage.

2. Antibody to HIV-1 (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

3. Anti Rabbit IgG Conjugate (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

4. Diluent for OPD tablets. Store at 2-8^{0}C. Note manufacturer's outdate.

5. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2-30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution fresh for each assay as follows:

   a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

   b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must be used within 60 minutes. OPD Substrate must not be exposed to strong light.
Do not cap tightly while dissolving.

6. Specimen Diluent containing Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

7. Stop Solution (1N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2-8^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/10, or other appropriate disinfectant.

2. Deionized or distilled water.

C. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

a. Just prior to set up, thaw 1 vial of each of the 5 concentrations.

b. Mix well and use.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes

Disposable reagent reservoirs
Vortex mixer
Centrifuge
Commander Dynamic Incubator (Abbott) capable of 40 ± 2^0C with rotation
Graduated cylinders and beakers
12 x 75 mm tubes
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well
Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm
Bead dispenser (Abbott)
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware

V. PROCEDURE

1. Create an EIA template in the virology data-management software (see software manual).

2. In an Abbott EIA reaction tray, dispense 50 µL of Specimen Diluent to each well.

3. Transfer 200 µL of the SQC, and patient specimens to the corresponding wells in the reaction tray.

4. Carefully dispense 1 bead to each testing well.

5. Cover the reaction tray using an adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 1 hour ± 5 minutes using an Abbott Dynamic Incubator with rotation.

6. Remove the cover from the reaction tray and discard. Wash each bead.

7. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 1 hour ± 5 minutes using an Abbott Dynamic Incubator with rotation.

8. Remove the cover from the reaction tray and discard. Wash each bead.

9. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 1 hour ± 5 minutes using an Abbott Dynamic Incubator with rotation.

10. Remove the cover from the reaction tray and discard. Wash each bead.

11. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

12. Add 1 mL of Stop Solution to all tubes.

13. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

When dispensing beads, remove cap from bead bottle, attach Bead Dispenser and dispense beads into wells of the reaction tray. Beads may also be dispensed using plastic forceps.

Do not splash liquid when tapping trays.

When transferring beads from wells to assay tubes, align inverted rack of orientated tubes over the reaction tray. Take care that well A1 aligns with tube A1! Press the tubes tightly over the

wells and invert tray and tubes together so the beads fall into corresponding tubes. Blot excess water from the top of the tube rack.

Dispense acid in same tube sequence as OPD Substrate Solution.

IX. REFERENCES

Abbott HIV-1 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Monoclonal
Immune Complex Dissociation

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The ICD HIV-1 antigen assay is a modification of the standard HIV-1 antigen assay. Circulating HIV-1 antigen binds to native HIV-1 antibody to form an immune complex and that is hidden from detection by the standard assay. The dissociation of the immune complex, accomplished by pH and heat, allows the p24 antigen to become detectable by the routine assay.

The Abbott HIVAG-1 Monoclonal Assay is an enzyme immunoassay (EIA) developed for detection uncomplexed HIV-1 p24 antigen. The Abbott HIVAG-1 Monoclonal Assay is a "sandwich" solid phase immunoassay that uses a polystyrene bead coated with human monoclonal antibody (Ab) to HIV-1 p24. If present, the viral antigen binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the beads. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long-term storage. Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis. Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay. Heat-inactivated specimens or specimens with obvious microbial

contamination are unacceptable. Avoid subjecting specimens to repeated freeze thaw cycles. Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in the Abbott ICD Preparation Kit include:

1. Reagent A (Glycine Reagent). Store at room temperature. Note manufacturer's outdate.

2. Reagent B (Tris Reagent). Store at room temperature. Note manufacturer's outdate.

3. Neutral Buffer.

   a. Prepare the Neutral Buffer by mixing 950 тL Glycine Reagent with 50 µL Tris Reagent.

   b. Prepare enough for each assay and discard unused portion.

B. Reagents included in the Abbott HIVAG-1 Monoclonal Kit:

1. Specimen Diluent containing Triton X-100. Store at 2 -8^{0}C. Note manufacturer's outdate

2. HIV-1 (Human) Monoclonal Antibody-coated Beads. Store at 2 -8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly for storage.

3. Antibody to HIV-1 (Rabbit). Store at 2 -8^{0}C. Note manufacturer's outdate.

4. Anti Rabbit IgG Conjugate (Rabbit). Store at 2 -8^{0}C. Note manufacturer's outdate.

5. Diluent for OPD tablets. Store at 2 -8^{0}C. Note manufacturer's outdate.

6. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2 -30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution fresh for each assay as follows:

   a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

   b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must be used within 60 minutes. OPD Substrate must not be exposed to strong light. Do not cap tightly while dissolving.

7. Stop Solution (1N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2-8^{0}C. Note manufacturer's outdate.

C. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

D. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

a. Just prior to set up, thaw 1 vial of each of the 5 concentrations.

b. Mix well and use.

2. ICD p24 Positive Control. Once reconstituted, store at 2-8^{0}C for up to 2 weeks.

a. Reconstitute the lyophilized ICD positive control with 2.0 mL of dH_2O.

b. Gently mix by inversion and allow 15 minutes for the reagent to go into solution.

c. Divide into aliquots. Use one aliquot for current assay and store others at -70 ^{0}C

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes
Disposable reagent reservoirs
Vortex mixer
Centrifuge
Incubator capable of 70 $\pm$ 1^{0}C
Commander Dynamic Incubator (Abbott) capable of 40 $\pm$ 2^{0}C with rotation
Graduated cylinders and beakers
12 x 75 mm tubes
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well
Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm
Bead dispenser (Abbott)
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware

V. PROCEDURE

A. ICD Specimen Setup

1. Create an ICD EIA template in the virology data-management software (see software manual).

2. Dispense 100 µL of each SQC, ICD Positive control or patient specimen as noted on the template into each well of an Abbott EIA reaction tray or 12 x 75 mm tubes. The ICD Positive control will be treated with ICD Reagent A or B as described in step 3., resulting in Treated ICD Positive Control, T100, and Untreated ICD Positive Control, U100.

3. Dispense 190 µL of Reagent A (glycine reagent) to each well except the U100 ICD Positive Control. To the U100 ICD Positive Control, add 190 µL Neutral Buffer. Gently mix the plate or tubes.

4. Carefully seal the plate with an adhesive plate cover, or cap tubes and incubate at 70 +/-1^{0}C for 10 minutes.

5. Remove the plate or tubes from the incubator. Dispense 10 µL of Reagent B (Tris reagent) to each well or tube except the U100 ICD Positive Control. Add 10 µL of Neutral Buffer to the U100 ICD Positive Control.

6. In a new Abbott EIA reaction tray, dispense 50 µL of Specimen Diluent to each well.

7. Transfer 200 µL of the samples prepared in step 2. through 5., (SQC, ICD Positive controls (U100 and T100) and patient specimens) to the corresponding wells in the new Abbott EIA reaction tray from step 6.

8. Proceed with the standard Abbott HIV-1 Antigen Assay

B. HIV Antigen Procedure

1. Carefully dispense 1 bead to each testing well.

2. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^{0}C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

3. Remove the cover from the reaction tray and discard. Wash each bead.

4. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^{0}C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

5. Remove the cover from the reaction tray and discard. Wash each bead.

6. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubble and incubate the plate at 40 $\pm$ 2^{0}C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

7. Remove the cover from the reaction tray and discard. Wash each bead.

8. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

9. Add 1 mL of Stop Solution to all tubes.

10. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of Stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and the Treated and Untreated ICD Positive Control, and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

When dispensing beads, remove cap from bead bottle, attach Bead Dispenser and dispense beads into wells of the reaction tray. Beads may also be dispensed using plastic forceps.

Do not splash liquid when tapping trays.

When transferring beads from wells to assay tubes, align inverted rack of orientated tubes over the reaction tray. Take care that well A1 aligns with tube A1! Press the tubes tightly over the wells and invert tray and tubes together so the beads fall into corresponding tubes. Blot excess water from the top of the tube rack.

Dispense acid in same tube sequence as OPD Substrate Solution.

IX. REFERENCES

Abbott HIV-1 Antigen Assay package insert and all references within.
Abbott ICD Prep Kit package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Monoclonal
Culture Supernatant Assay

I. PRINCIPLE

The Abbott HIVAG-1 Monoclonal Assay is an enzyme immunoassay (EIA) developed for the detection of HIV-1 p24 antigen. It may be used to detect the presence of p24 antigen in the supernatant of a HIV-1 culture. The Abbott HIVAG-1 Monoclonal Assay is a "sandwich" solid phase immunoassay that uses polystyrene beads coated with a human polyclonal antibody (Ab) to HIV-1 p24. If present, the viral p24 antigen (Ag) binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the bead. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of a known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Aliquots of culture supernatant should be collected and frozen at -20^{0}C until tested.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all samples to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. The following reagents are included in the Abbott HIVAG-1 Monoclonal Kit. Kit reagents may be used at room temperature or cold unless otherwise stated in reagent preparation.

1. HIV-1 (Human) Monoclonal Antibody-coated Beads. Store at 2-8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly.

2. Antibody to HIV-1 (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

3. Anti Rabbit IgG Conjugate (Goat). Store at 2-8^{0}C. Note manufacturer's outdate.

4. Diluent for OPD tablets. Store at 2-8^{0}C. Note manufacturer's outdate.

5. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2-30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution as follow:

   a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

   b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

   c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

      Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must be used within 60 minutes. OPD Substrate must not be exposed to strong light. Do not cap tightly while dissolving.

6. Specimen Diluent containing Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

7. Stop Solution (1N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2-8^0. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/10 or other appropriate disinfectant.

2. Deionized or distilled water.

3. Supernatant from uninfected (HIV negative) donor PBMC culture.

C. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA 400 pg/mL Standard. Store one vial designated as "Working Standard" at 2 -8^{0}C for up to one month. Other vials should be stored at -70^{0}C or lower. Dilutions for standard curve should be made fresh for each assay as follows:

   a. Make four 5-fold dilutions of the VQA 400 pg/mL Standard using VQA Diluent for dilutions.

   b. Pipette 500 µL of VQA Diluent into four tubes. Pipette 500 µL of VQA 400 pg/mL Standard into the first tube and mix. Changing tips, pipette 500 µL of the first dilution into the second tube of diluent and mix.

   c. Continue until four dilutions are made.

   Note: These dilutions can be prepared using larger volumes. Each dilution would then be dispensed into aliquots which are frozen at -70^{0}C until use.

2. VQA QC (Media Quality Control). A set of three concentrations. One vial of each concentration may be stored at 2-8^{0}C for up to one month. Other vials should be stored at -70^{0}C or lower.

3. VQA Diluent. One bottle of diluent may be stored at 2 -8^{0}C for up to one month. Other bottles should be stored at -70^{0}C or lower.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes
Disposable reagent reservoirs
Vortex mixer
Commander Dynamic Incubator (Abbott) or other incubator capable of 40 $\pm$ 2^{0}C
Graduated cylinders and beakers
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well

Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm.
Bead dispenser (Abbott)-optional
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware

V. PROCEDURE

1. Create an EIA template in the virology data-management software (see software manual).

2. In an Abbott EIA reaction tray, dispense 50 µL of Specimen Diluent to each well except the wells receiving the V100.

3. Transfer 200 µL of the standard dilutions, Media QC, and patient specimens to the corresponding wells in the reaction tray.

4. Carefully dispense 1 bead to each testing well.

5. Cover the reaction tray using an adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^0C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

6. Remove the cover from the reaction tray and discard. Wash each bead.

7. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^0C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

8. Remove the cover from the reaction tray and discard. Wash each bead.

9. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^0C for 1 hour $\pm$ 5 minutes using an Abbott Dynamic Incubator with rotation.

10. Remove the cover from the reaction tray and discard. Wash each bead.

11. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

12. Add 1 mL of Stop Solution to all tubes.

13. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA standard concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA standards, and the VQA media QC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

When dispensing beads, remove cap from bead bottle, attach Bead Dispenser and dispense beads into wells of the reaction tray. Beads may also be dispensed using plastic forceps.

Do not splash liquid when tapping trays.

When transferring beads from wells to assay tubes, align inverted rack of orientated tubes over the reaction tray. Take care that well A1 aligns with tube A1! Press the tubes tightly over the wells and invert tray and tubes together so the beads fall into corresponding tubes. Blot excess water from the top of the tube rack.

Dispense acid in same tube sequence as OPD Substrate Solution.

IX. REFERENCES

Abbot HIV-1 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Polyclonal
Direct Serum Assay

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The Abbott HIVAG-1 Polyclonal Assay is an enzyme immunoassay (EIA) developed for detection uncomplexed HIV-1 p24 antigen. The Abbott HIVAG-1 Monoclonal Assay is a "sandwich" solid phase immunoassay that uses a polystyrene bead coated with human polyclonal antibody (Ab) to HIV-1 p24. If present, the viral antigen binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the beads, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the bead. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of known a HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C to -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. The following reagents are included in the Abbott HIVAG-1 Polyclonal Kit. Kit reagents may be used at room temperature or cold unless otherwise stated in reagent preparation.

1. HIV-1 (Human) Polyclonal Antibody-coated Beads. Store at 2-8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly for storage.

2. Antibody to HIV-1 (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

3. Anti Rabbit IgG Conjugate (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

4. Diluent for OPD tablets. Store at 2-8^{0}C. Note manufacturer's outdate.

5. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2-30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution fresh for each assay as follows:

   a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

   b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

   c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

      Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must

be used within 60 minutes. OPD Substrate must not be exposed to strong light.
Do not cap tightly while dissolving.

6. Specimen Diluent containing Triton X-100. Store at 2 -8^{0}C. Note manufacturer's outdate.

7. Stop Solution (1N H_2SO_4). Store at 2 -30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2 -8^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/10 or other appropriate disinfectant.

2. Deionized or distilled water.

C. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

a. Just prior to set up, thaw 1 vial of each of the 5 concentrations.

b. Mix well and use.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes
Disposable reagent reservoirs
Vortex mixer

Centrifuge
Commander Dynamic Incubator (Abbott) capable of 40 ± 2^0C with rotation
Graduated cylinders and beakers
12 x 75 mm tubes
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well
Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm
Bead dispenser (Abbott)
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware

V. PROCEDURE

1. Create an EIA template in the virology data-management software (see software manual).

2. In an Abbott EIA reaction tray, dispense 20 µL of Specimen Diluent to each well.

3. Dispense 200 µL Abbott negative control into the first 3 wells of the reaction tray.

4. Transfer 200 µL of the SQC and patient specimens into the corresponding wells in the reaction tray according template.

5. Carefully dispense 1 bead into each testing well, avoiding splashes.

6. Cover the reaction tray using an adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at room temperature (15-30^{0}C) for 16-20 hours.

7. Remove the cover from the reaction tray and discard. Wash each bead.

8. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 4 hours ± 10 minutes.

9. Remove the cover from the reaction tray and discard. Wash each bead.

10. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 1 hour ± 10 minutes.

11. Remove the cover from the reaction tray and discard. Wash each bead.

12. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

13. Add 1 mL of Stop Solution to all tubes.

14. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of Stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA standard concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

When dispensing beads, remove cap from bead bottle, attach Bead Dispenser and dispense beads into wells of the reaction tray. Beads may also be dispensed using plastic forceps.

Do not splash liquid when tapping trays.

When transferring beads from wells to assay tubes, align inverted rack of orientated tubes over the reaction tray. Take care that well A1 aligns with tube A1! Press the tubes tightly over the wells and invert tray and tubes together so the beads fall into corresponding tubes. Blot excess water from the top of the tube rack.

Dispense acid in same tube sequence as OPD Substrate Solution.

IX. REFERENCES

Abbott HIV-1 Antigen Assay package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Polyclonal
Immune Complex Dissociation

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The ICD HIV-1 antigen assay is a modification of the standard HIV-1 antigen assay. Circulating HIV-1 antigen binds to native HIV-1 antibody to form an immune complex and that is hidden from detection by the standard assay. The dissociation of the immune complex, accomplished by pH and heat, allows the p24 antigen to become detectable by the routine assay.

The Abbott HIVAG-1 Polyclonal Assay is an enzyme immunoassay (EIA) developed for detection uncomplexed HIV-1 p24 antigen. The Abbott HIVAG-1 Polyclonal Assay is a "sandwich" solid phase immunoassay that uses a polystyrene bead coated with human polyclonal antibody (Ab) to HIV-1 p24. If present, the viral antigen binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the beads. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis. Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay. Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Avoid subjecting specimens to repeated freeze thaw cycles. Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in the Abbott ICD Preparation Kit include:

1. Reagent A (Glycine Reagent). Store at room temperature. Note manufacturer's outdate.

2. Reagent B (Tris Reagent). Store at room temperature. Note manufacturer's outdate.

3. Neutral Buffer.

   a. Prepare the Neutral Buffer by mixing 950 µL Glycine Reagent with 50 µL Tris Reagent.

   b. Prepare enough for each assay and discard unused portion.

B. Reagents included in the Abbott HIVAG-1 Polyclonal Kit:

1. Specimen Diluent containing Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

2. HIV-1 (Human) Polyclonal Antibody-coated Beads. Store at 2-8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly for storage.

3. Antibody to HIV-1 (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

4. Anti Rabbit IgG Conjugate (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

5. Diluent for OPD tablets. Store at 2-8^{0}C. Note manufacturer's outdate.

6. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2 -30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution fresh for each assay as follows:

a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No. of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must be used within 60 minutes. OPD Substrate must not be exposed to strong light. Do not cap tightly while dissolving.

7. Stop Solution (1N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2-8^{0}C. Note manufacturer's outdate.

C. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/10 or other appropriate disinfectant.

2. Deionized or distilled water.

D. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

a. Just prior to set up, thaw 1 vial of each of the 5 concentrations.

b. Mix well and use.

2. ICD p24 Positive Control. Once reconstituted, store at 2-8^{0}C for up to 2 weeks.

   a. Reconstitute the lyophilized ICD positive control with 2.0 mL of dH_2O.

   b. Gently mix by inversion and allow 15 minutes for the reagent to go into solution.

   c. Divide into aliquots. Use one aliquot for current assay and store others at -70^{0}C.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes
Disposable reagent reservoirs
Vortex mixer
Centrifuge
Incubator capable of 70 $\pm$ 1^{0}C
Commander Dynamic Incubator (Abbott) capable of 40 $\pm$ 2^{0}C with rotation
Graduated cylinders and beakers
12 x 75 mm tubes
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well
Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm
Bead dispenser (Abbott)
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware.

V. PROCEDURE

A. ICD Specimen Setup

1. Create an ICD EIA template in the virology data-management software (see software manual).

2. Dispense 100 µL of each SQC, ICD Positive control or patient specimen as noted on the template into each well of an Abbott EIA reaction tray or 12 x 75 mm tubes. The ICD Positive control will be treated with ICD Reagent A or B as described in step 3., resulting in Treated ICD Positive Control, T100, and Untreated ICD Positive Control, U100.

3. Dispense 190 µL of Reagent A (glycine reagent) to each well except the U100 ICD Positive Control. To the U100 ICD Positive Control, add 190 µL Neutral Buffer. Gently mix the plate or tubes.

4. Carefully seal the plate with an adhesive plate cover, or cap tubes and incubate at 70 +/-1^{0}C for 10 minutes.

5. Remove the plate or tubes from the incubator. Dispense 10 µL of Reagent B (Tris reagent) to each well or tube except the U100 ICD Positive Control. Add 10 µL of Neutral Buffer to the U100 ICD Positive Control.

6. In a new Abbott EIA reaction tray, dispense 50 µL of Specimen Diluent to each well.

7. Transfer 200 µL of the samples prepared in step 2. through 5., (SQC, ICD Positive controls (U100 and T100) and patient specimens) to the corresponding wells in the new Abbott EIA reaction tray from step 6.

8. Proceed with the standard Abbott HIV-1 Antigen Assay

B. HIV Antigen Procedure

1. Carefully dispense 1 bead into each testing well, avoiding splashes.

2. Cover the reaction tray using an adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at room temperature (15-30^{0}C) for 16-20 hours.

3. Remove the cover from the reaction tray and discard. Wash each bead.

4. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^{0}C for 4 hours $\pm$ 10 minutes.

5. Remove the cover from the reaction tray and discard. Wash each bead.

6. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 ± 2^0C for 1 hour ± 10 minutes.

7. Remove the cover from the reaction tray and discard. Wash each bead.

8. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 ± 2 minutes.

9. Add 1 mL of Stop Solution to all tubes.

10. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of Stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and the Treated and Untreated ICD Positive Control and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

When dispensing beads, remove cap from bead bottle, attach Bead Dispenser and dispense beads into wells of the reaction tray. Beads may also be dispensed using plastic forceps.

Do not splash liquid when tapping trays.

When transferring beads from wells to assay tubes, align inverted rack of orientated tubes over the reaction tray. Take care that well A1 aligns with tube A1! Press the tubes tightly over the wells and invert tray and tubes together so the beads fall into corresponding tubes. Blot excess water from the top of the tube rack.

Dispense acid in same tube sequence as OPD Substrate Solution.

IX. REFERENCES

Abbott HIV-1 Antigen Assay package insert and all references within.
Abbott ICD Prep Kit package insert and all references within.

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ANTIGENS
Abbott HIVAG-1 Polyclonal
Culture Supernatant Assay

I. PRINCIPLE

The Abbott HIVAG-1 Polyclonal Assay is an enzyme immunoassay (EIA) developed for the detection of HIV-1 p24 antigen. It may be used to detect the presence of p24 antigen in the supernatant of a HIV-1 culture. The Abbott HIVAG-1 Polyclonal Assay is a "sandwich" solid phase immunoassay that uses polystyrene beads coated with a human polyclonal antibody (Ab) to HIV-1 p24. If present, the viral p24 antigen (Ag) binds to the antibody coated bead. Following a wash step, rabbit antibody to HIV-1 is then incubated with the beads and binds to the HIV-1 Ag. Goat antibody to rabbit IgG conjugated with horseradish peroxidase (anti-Rabbit IgG: HRPO) is incubated with the beads and binds to rabbit antibody. Next o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow-orange color develops in proportion to the amount of HIV-1 antigen bound to the bead. The quantity of HIV-1 antigen in a specimen is determined by comparing its absorbance with that of a known HIV-1 p24 antigen standard curve.

II. SPECIMEN REQUIREMENTS

Aliquots of culture supernatant should be collected and frozen at -20^{0}C until tested. Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable. Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all samples to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. The following reagents are included in the Abbott HIVAG-1 Polyclonal Kit. Kit reagents may be used at room temperature or cold unless otherwise stated in reagent preparation.

1. HIV-1 (Human) Polyclonal Antibody-coated Beads. Store at 2-8^{0}C. Note manufacturer's outdate. Replace desiccant after use and cap tightly for storage.

2. Antibody to HIV-1 (Rabbit). Store at 2-8^{0}C. Note manufacturer's outdate.

3. Anti Rabbit IgG Conjugate (Goat). Store at 2-8^{0}C. Note manufacturer's outdate.

4. Diluent for OPD tablets. Store at 2-8^{0}C. Note manufacturer's outdate.

5. OPD Tablets (o-Phenylenediamine-2HCL). Store at 2-30^{0}C. Note manufacturer's outdate. Prepare OPD Substrate Solution as follow:

   a. Bring OPD tablets and diluent to room temperature before use. Do not open tablet bottle until it reaches room temperature.

   b. Within 5-60 minutes of use, prepare sufficient OPD Substrate Solution by dissolving the OPD Tablets in the OPD Diluent. See chart below.

No. Of Beads	Tablets	Diluent (mL)
13	1	5
28	2	10
43	3	15
58	4	20
73	5	25
88	6	30
103	7	35

   c. Just prior to dispensing, swirl the container gently to obtain a homogenous solution.

      Note: Do not use an OPD tablet that is broken. Transfer tablets with non-metallic forceps into a metallic free bottle. OPD Substrate solution must be used within 60 minutes. OPD Substrate must not be exposed to strong light. Do not cap tightly while dissolving

6. Specimen Diluent containing Triton X-100. Store at 2-8^{0}C. Note manufacturer's outdate.

7. Stop Solution (1N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

8. Negative Control. Store at 2-8^{0}C. Note manufacturer's outdate.

B. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

C. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

1. VQA 400 pg/mL Standard. Store one vial designated as "Working Standard" at 2 -8^{0}C for up to one month. Other vials should be stored at -70^{0}C or lower. Dilutions for standard curve should be made fresh for each assay as follows:

   a. Make four 5-fold dilutions of the VQA 400 pg/mL Standard using VQA Diluent for dilutions.

   b. Pipette 500 l of VQA Diluent into four tubes. Pipette 500 l of VQA 400 pg/mL Standard into the first tube and mix. Changing tips, pipette 500 l of the first dilution into the second tube of diluent and mix.
   c. Continue until four dilutions are made.

2. VQA QC (Media Quality Control). A set of three concentrations. One vial of each concentration may be stored at 2-8^{0}C for up to one month. Other vials should be stored at -70^{0}C or lower.

3. VQA Diluent. One bottle of diluent may be stored at 2-8^{0}C for up to one month. Other bottles should be stored at -70^{0}C or lower.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Reaction Trays (Abbott)
Assay Tubes (Abbott)
Cover seals (Abbott)
Micropipet(s) capable of delivering 20, 50, 180 µL volumes
Precision pipettes, or similar equipment to deliver 200 µL, 300 µL ,and 1 mL
Disposable pipette tips suitable for the above pipettes
Disposable serological pipettes
Disposable reagent reservoirs
Vortex mixer
Commander Dynamic Incubator (Abbott) or other incubator capable of 40 $\pm$ 2^{0}C
Graduated cylinders and beakers
Washing device for washing beads such as Quickwash® or Pentawash® II with vacuum source and a double trap for retaining the aspirate, and capable of delivering a total rinse volume of 4-6 mL per well
Quantumatic™ or Quantum Analyzer™ or spectrophotometer able to read absorbance at 492 nm
Bead dispenser (Abbott)-optional
Non-metallic forceps
Metal free container for OPD Substrate Solution can be plastic or acid washed glassware

V. PROCEDURE

1 Create an EIA template in the virology data-management software (see software manual).

2. In an Abbott EIA reaction tray, dispense 20 µL of Specimen Diluent to each well except the wells receiving the VQA standard dilutions and QC samples.

3. Dispense 20 µL of Abbott negative control to each well except the wells receiving the VQA standard dilutions and QC samples.

4. Dispense 200 µL uninfected donor culture supernatant into the first 3 wells of the reaction tray.

5. Transfer 200 µL of the VQA standard dilutions, QC samples and patient specimens into the corresponding wells in the reaction tray according template.

6. Carefully dispense 1 bead into each testing well, avoiding splashes.

7. Cover the reaction tray using an adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at room temperature (15-30^{0}C) for 16-20 hours.

8. Remove the cover from the reaction tray and discard. Wash each bead.

9. Add 200 µL Antibody Solution to all testing wells. Cover the reaction tray using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^{0}C for 4 hours $\pm$ 10 minutes.

10. Remove the cover from the reaction tray and discard. Wash each bead.

11. Add 200 µL of Conjugate Solution to all testing wells. Cover the plate using a new adhesive plate cover. Gently tap the tray to remove trapped air bubbles and incubate the plate at 40 $\pm$ 2^{0}C for 1 hour $\pm$ 10 minutes.

12. Remove the cover from the reaction tray and discard. Wash each bead.

13. Immediately transfer the beads to a properly identified box of assay tubes. Add 300 µL of OPD substrate solution into 2 empty tubes (substrate blanks) and then into all tubes containing a bead. Cover the tubes to prevent exposure to intense light. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

14. Add 1 mL of Stop Solution to all tubes.

15. Blank spectrophotometer with one of the substrate blank tubes at 492 nm. Read the absorbance of each tube at 492 nm within 2 hours of the addition of Stop Solution.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA standard concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA standards, and the VQA media QC and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

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